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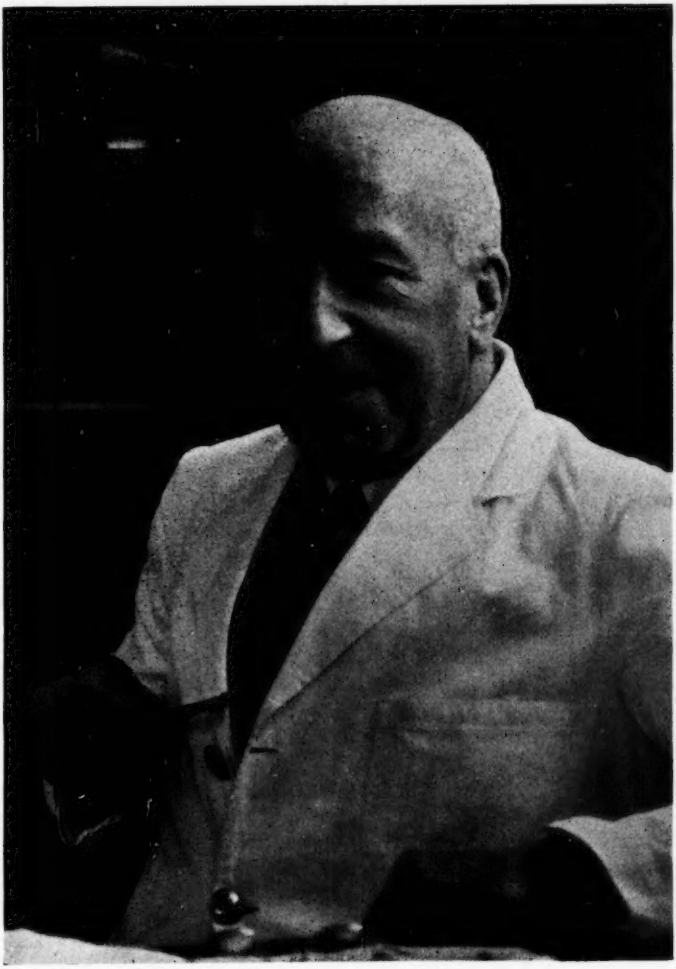

Paul Fildes

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

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# LINKED ANTI-A AND ANTI-B ANTIBODIES FROM GROUP O SERA

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IT is accepted that antigen molecules can have more than one combining site, but the existence of a number of combining sites on an antibody molecule is unproven. Heidelberger and Kendall (1935), Kabat and Heidelberger (1937) and Marrack (1938) considered the precipitate formed in the precipitin reaction to be a network of antigen and antibody molecules, each antigen bound to several antibodies and each antibody molecule to two or more antigen molecules, thus postulating antibodies with several combining sites. Meyer and Pic (1937) offer evidence for the presence of specifically different combining groups in a single antibody. They studied sera from rabbits immunised against aqueous extracts of tubercle bacilli; these sera reacted with both the lipids and the polysaccharide of the tubercle bacilli. By preparation of antibody solutions from lipid or polysaccharide absorbed on kaolin, they obtained from either preparation a solution which reacted with both antigens. They concluded that this was due to the presence in the rabbit sera of bivalent antibodies with a combining site for each of the two antigens.

More recent work tends to favour the idea of univalent antibodies. Landsteiner and van der Scheer (1938) prepared immune sera to azoproteins with two structurally different active groups in the same molecule, viz., succinic and phenylarsenic acids, and to glycine and leucine. When the immune sera were tested by absorption with one or other partial antigens there was no reduction in reactivity with the antigen containing the other determinant group. Thus these authors conclude that there is no evidence for the formation in these sera of antibodies having two different combining sites. Haurowitz and Schwerin (1942, 1943) also conclude from results obtained in precipitation experiments that immunisation with a complex antigen containing two kinds of determinant groups does not produce antibodies with both kinds of specificity.

The present work was the outcome of the observation that when A or B red cells absorbed and subsequently gave up agglutinins originating from human group O sera (containing both anti-A and anti-B agglutinins) the antibody solu-

tion obtained from red cells of either group agglutinated both A and B red cells. In other words, an agglutinin solution was prepared from group A cells which, as well as agglutinating group A cells, agglutinated cells of group B, and *vice versa*.

The simplest explanation of this finding is that at least some of the globulin molecules in group O serum possess both anti-A and anti-B specific combining sites, and so can be removed by either A or B cells.

The fact that antibodies absorbed from group O sera by A or B cells and subsequently recovered, agglutinated both A and B red cells, has been observed by Landsteiner and Witt (1926), Thomsen and Worsaae (1929), and Fischer and Hahn (1935). The agglutination of heterologous cells was attributed to a non-specific reaction, although Landsteiner and Witt suggested that group O sera as well as containing $\alpha$ and $\beta$ also contain some antibody units which might be written $\alpha + \beta$. Evidence that other blood group antibody molecules may possess combining sites for two different antigens is provided by the work of Race, Sanger and Lawler (1948) with the antibodies anti-C and anti-$C^w$ of the Rh system.

The following experiments were designed to establish the existence in human group O serum of antibody molecules having two specifically different combining sites, anti-A and anti-B.

## MATERIALS AND METHODS.

*Red cells.*—The group A and group B red cells used for titration of antibodies and preparation of antibody solutions were almost always taken from the same two donors throughout. Blood was obtained by venepuncture and placed in a sodium citrate glucose mixture. It could then be used for experiments on two consecutive days, but was not kept longer. Before use the red cells were washed three times in 0·85 per cent saline. Packed cells were used for the preparation of antibody solutions and an approximately 2 per cent suspension in saline for all titrations except haemolysin titres, for which an approximately 5 per cent suspension was used.

*Anti-human-globulin serum for Coombs' tests.*—A very potent anti-human globulin rabbit serum, kindly provided by Professor Marrack, was used throughout the investigation. This was used at a dilution in saline of 1 : 100.

*Purified A and B group-specific substances.*—A and B group-specific substances prepared from pseudomucinous ovarian cysts (Morgan and van Heyningen, 1944) according to the method described by King and Morgan (1944) were supplied by Dr. Morgan, and were used for inhibition tests and for intramuscular injection into group O volunteers.

*Complement for haemolysin tests.*—Dried guinea-pig complement was used with some success, although usually fresh human complement was found to be more satisfactory.

*Titration of anti-A and anti-B antibodies.*—The method used for titrating antibodies was essentially as described by Taylor and Ikin (1939). Doubled dilutions of test serum were made in saline with a Pasteur pipette graduated to deliver a constant volume (about 0·03 ml.). To each of the dilutions an equal volume of a 2 per cent suspension of appropriate red cells was added and the tubes shaken. The titrations were made in duplicate and allowed to stand at room temperature for 2–3 hours, after which the contents of the tubes were examined for agglutination both macroscopically and microscopically. The method of scoring the

degree of agglutination is shown in the key to Table I. The presence of evenly distributed clumps of three or more cells was accepted as evidence of agglutination. The titre of a serum was expressed as the reciprocal of the highest dilution of serum causing agglutination.

*Titration by anti-globulin technique.*—The anti-human globulin test of Coombs, Mourant and Race (1945) was developed for the detection of a type of Rh antibody which sensitized red cells suspended in saline without causing them to agglutinate. Red cells sensitized by these Rh antibodies and washed free from serum were agglutinated on exposure to the serum of a rabbit which had been previously immunised against human globulin, or against human whole serum.

This test is applicable in certain circumstances to the antibodies of the ABO system (Boorman and Dodd, 1946), and has proved invaluable in the present work, where antibody is apparently concerned which sensitizes the red cell more readily than it causes agglutination.

The titrations were carried out as described and kept at room temperature for at least 1 hour. The red cells in each tube were then freed from serum by two washings in saline, after which the cell sediment from each tube was mixed on a slide with one drop of suitably diluted anti-human-globulin serum freshly made up for each batch of tests. The slides were left on the bench for an interval of from 5–10 min. with occasional rocking and then examined for agglutination. The degree of agglutination was scored in the usual manner. When Coombs' titrations were performed on antibody eluted from red cells the washing stage was omitted.

*Titration of anti-A and anti-B haemolysins.*—Sera containing haemolysins were titrated using the Pasteur pipette technique and guinea-pig complement or fresh human serum as diluent instead of 0·85 per cent saline. It was found that the best results were obtained when the sera were titrated within 3 hours of venepuncture without the addition of complement. This finding suggested that the complement in the test serum itself was more efficient in the production of haemolysis than added complement of either human or animal origin. After the addition of one unit volume of 5 per cent red cells to each tube, the tests were incubated for approximately two hours at 37°. They were then removed from the incubator, shaken and allowed to stand at room temperature until the red cells had sedimented or the tubes were centrifuged. The degree of haemolysis in each tube was then read.

*Elution of antibody from the red cell surface.*—The method was one adapted from Landsteiner and Miller (1925). In the first experiments equal parts of a group O serum and packed washed red cells of group A and B respectively were mixed and left at room temperature for at least one hour. Later it was found that the potency of the antibody finally recovered was improved if the red cell volume was reduced and the absorption stage carried out at 4°. A mixture of 10 drops of packed washed cells and 2 ml. of serum was therefore placed in the ice chest to absorb for 1 hour.

The cells, which were usually strongly agglutinated, were then carefully washed at 4°. About 8 ml. of saline was used for each washing and the number of washings given related to some extent to the potency of the original group O serum, the minimum number being four and the maximum six. After each washing the agglutinated cells, which were often compacted into a solid mass by the action of the centrifuge, were broken down as completely as possible by vigorous shaking.

In this way an attempt was made to ensure that no antibody remained caught up mechanically in the agglutinated cell mass. In each experiment the last washing of the red cells was kept and tested for antibody together with the eluted antibody. After the cells were thoroughly washed 2 ml. saline was added, and after thorough mixing the suspension was placed in a water-bath at 56° for 10 minutes. During this time the suspension was continually agitated. The tubes were then transferred quickly to centrifuge cups previously heated to 56° and lagged with cotton-wool soaked in water at 56°. They were then centrifuged strongly for a short time and the supernatant fluid (the eluate) which contained the liberated antibody removed as quickly as possible from the cells. Antibody solutions thus prepared were always tested for antibody as far as possible on the day on which they were prepared, because it was found that they underwent a certain degree of deterioration, even if stored at — 40°.

## EXPERIMENTAL.

*Series* 1 : *Antibody eluted from A and B cells after these had been allowed to absorb antibody from immune group O sera.*

Twenty-two cases were investigated ; of these 12 sera contained immune anti-A antibody and 10 immune anti-B antibody. Eight of the sera containing immune A antibodies were from group O mothers who had been carrying a foetus of group A, while 3 were from group O individuals injected with purified A group specific substance, and 1 was from a group O woman who had received an incompatible transfusion of 500 ml. of group A blood. Of the 10 sera containing immune anti-B antibodies 6 were from group O mothers carrying a group B foetus, and 4 were from individuals who had received an intramuscular injection of group specific substance B. The individuals immunised by either A or B

TABLE I.—*Typical Protocol showing Details of Titrations.*

| | Original serum. | | | | Antibody solutions. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | From A cells. | | | | From B cells. | | | |
| Serial dilutions. | Anti-A. | | Anti-B. | | Against A. | | Against B. | | Against A. | | Against B. | |
| | *S* | *Co* | *S* | *Co* | *S* | *Co* | *S* | *Co* | *S* | *Co* | *S* | *Co* |
| 1 : 1 | c | c | c | c | v | v | v | v | v | v | v | v |
| 1 : 2 | c | c | c | c | + + | v | v | v | + + | v | + | v |
| 1 : 4 | v | v | v | v | + + | v | + | v | + | v | (+) | v |
| 1 : 8 | v | v | v | v | (+) | (+) | gw | v | (+) | v | w | v |
| 1 : 16 | + + | v | + + | v | w | w | w | + | w | v | − | v |
| 1 : 32 | + + | v | + + | v | − | w | − | w | − | (+) | .. | + + |
| 1 : 64 | + | v | + | v | .. | − | .. | − | .. | w | .. | (+) |
| 1 : 128 | + | v | + | v | .. | .. | .. | .. | .. | − | .. | (+) |
| 1 : 256 | (+) | v | w | + + | .. | .. | .. | .. | .. | .. | .. | − |
| 1 : 512 | gw | + + | − | w | | | | | | | | |
| 1 : 1000 | w | w | .. | − | | | | | | | | |
| 1 : 2000 | − | − | | | | | | | | | | |
| 1 : 4000 | | | | | | | | | | | | |

*S* = agglutinin titre. *Co* = Coombs' titre.

Read macroscopically : c = complete agglutination ; surrounding fluid clear. *V* = visual agglutination ; surrounding fluid pink.

Read microscopically : + + = very large clumps—few free cells. + = large clumps—more free cells. (+) = smaller clumps with many free cells. gw = slightly smaller clumps with many free cells. w = evenly distributed clumps of 2–5 cells. − = no agglutination.

TABLE II.—*Anti-A and Anti-B Titres of 22 Immune Group O Sera, together with Titres of Antibody Solutions Prepared from these Sera.*

| Cases. | Immune antibody. | Titre of original serum. | | | | Titre of antibody solution from A cells. | | | | Titre of antibody solution from B cells. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Against A cells. | | Against B cells. | | Against A cells. | | Against B cells. | | Against A cells. | | Against B cells. | |
| | | S | Co | S | Co | S | Co | S | Co | S | Co | S | Co |
| 1. Jasper | Anti-A | 1,000 | 16,000 | 64 | 128 | 32 | 4,000 | – | 2 | 16 | 1,000 | 16 | 8 |
| 2. Grayson | Anti-B | 512 | 4,000 | 1,000 | 8,000 | 32 | 64 | 32 | 128 | 2 | 4 | 2 | 32 |
| 3. Dark | Anti-A | 512 | 1,000 | 64 | 256 | – | 64 | – | 32 | 4 | 128 | 1 | 8 |
| 4. Sparks | „ | 32,000 | 128,000 | 1,000 | 2,000 | 128 | 8,000 | 4 | 8 | 64 | 16,000 | 4 | 8 |
| 5. Taylor | „ | 4,000 | 64,000 | 32 | 128 | 16 | 2,000 | – | 4 | 32 | 512 | 1 | 512 |
| 6. Cooper | Anti-B | 256 | 1,000 | 1,000 | 8,000 | 32 | 32 | 64 | 128 | 32 | 32 | 32 | 32 |
| 7. Golding | Anti-A | 2,000 | 8,000 | 256 | 512 | 32 | 32 | – | 4 | 16 | 32 | 4 | 16 |
| 8. Prince | Anti-B | 64 | 128 | 512 | 1,000 | 4 | 8 | 1 | 16 | 1 | 8 | 2 | 8 |
| 9. McRae | Anti-A | 4,000 | 16,000 | 1,000 | 1,000 | 2,000 | 2,000 | 64 | 128 | 64 | 128 | 16 | 1,000 |
| 10. Scott | „ | 4,000 | 32,000 | 128 | 1,000 | 64 | 1,000 | 1 | 8 | 2 | 8 | 16 | 32 |
| 11. Pearson | „ | 2,000 | 4,000 | 64 | 128 | 32 | 64 | 2 | 128 | 16 | 64 | 8 | 16 |
| 12. Young | Anti-B | 128 | 512 | 1,000 | 4,000 | 32 | 64 | 64 | 256 | 4 | 8 | 1,000 | 2,000 |
| 13. Howe | Anti-A | 2,000 | 8,000 | 16 | 32 | 32 | 128 | – | – | 8 | 64 | 8 | 128 |
| 14. Tysson | Anti-B | 256 | 2,000 | 1,000 | 4,000 | 32 | 256 | 8 | 32 | 4 | 16 | 64 | 1,000 |
| 15. Zeitlin | „ | 1,000 | 1,000 | 4,000 | 8,000 | 4 | 16 | 8 | 64 | 2 | 8 | 64 | 256 |
| 16. Sharp | Anti-A | 1,000 | 1,000 | 256 | 512 | 8 | 16 | 16 | 32 | 16 | 32 | 8 | 128 |
| 17. Butcher | Anti-B | 128 | 32,000 | 16,000 | 32,000 | 64 | 128 | 64 | 128 | 32 | 64 | 64 | 256 |
| 18. Adams | Anti-A | 512 | 16,000 | 64 | 256 | 128 | 512 | 4 | 128 | 128 | 256 | 16 | 256 |
| 19. McTrustery | „ | 4,000 | 512,000 | 256 | 512 | 128 | 8,000 | 8 | 16 | 128 | 256 | 16 | 32 |
| 20. Gore | Anti-B | 1,000 | 2,000 | 2,000 | 8,000 | 8 | 64 | 32 | 256 | 1 | 1 | 64 | 1,000 |
| 21. Sterile | „ | 512 | 8,000 | 2,000 | 32,000 | 128 | 256 | 128 | 256 | 2 | 8 | 64 | 1,000 |
| 22. Fitzgerald | „ | 512 | 2,000 | 8,000 | 16,000 | 64 | 512 | 8 | 64 | 2 | 32 | 32 | 512 |

TABLE III.—*Results Proving that Anti-A is Present in Antibody Solutions Prepared from Group B Cells, and Anti-B in Antibody Solutions Prepared from Group A Cells.*

| Case | Titre of antibody solution from A cells after neutralization with A substance. Against A cells. | Against B cells. | Titre of antibody solution from B cells after neutralisation with B substance. Against A cells. | Against B cells. |
|---|---|---|---|---|
| 17. Butcher | – | 8 | .. | .. |
| 9. McRae | .. | .. | 16 | – |
| 18. Adams | .. | .. | 2 | – |
| 19. McTrustery | .. | .. | Not tested | Not tested |
| 20. Gore | Not tested | Not tested | .. | .. |

| Case. | Tests against random Group A samples. 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Tests against random Group B samples. 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17. Butcher | – | – | – | – | – | – | – | – | – | – | w | w | + | + | + | + | + | + | + | + |
| 9. McRae | v | v | v | v | v | v | v | v | v | v | – | – | – | – | – | – | – | – | – | – |
| 18. Adams | + | + | w | + | + | (+) | + | + | + | + | – | – | – | – | – | – | – | – | – | – |
| 19. McTrustery | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | – | – | – | – | – | – | – | – | – | – |
| 20. Gore | – | – | – | – | – | – | – | – | – | – | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

purified substance received one dose of approximately 1·5 mg. of group specific substance by intramuscular injection. After eight to ten days a bleeding was obtained, and the immune serum thus obtained was used in these experiments.

The eluted antibody preparations were titrated against A and B red cells and examined also by the Coombs' technique. The specimens were also tested for their capacity to agglutinate group O cells.

*Results.*—A typical protocol is shown in Table I. This shows results of titrating antibody solutions (eluates) made from A and B cells which had absorbed antibody from immune group O sera. The results show that the antibody solution prepared from either group A or group B red cells is able to agglutinate *both* types of red cell. This is particularly well demonstrated when the Coombs' titration technique is used. Only a negligible quantity of antibody was present in the saline of the final washing. Results of similar experiments with the 22 immune group O sera are summarised in Table II. In every instance the recovered antibody agglutinates the heterologous cells. This is true whether the antigenic stimulus is afforded by a foetus *in utero*, blood transfusion (1 case only), or injection of group specific substances. None of the antibody solutions caused agglutination of group O cells.

*Comment.*—These findings are best explained by postulating the presence of both anti-A and anti-B antibodies in the antibody solutions whether these are derived from A or B red cells. As mentioned above, this could occur if there existed in the group O sera certain globulin molecules which possessed both anti-A and anti-B combining groups, thus enabling them to be removed from solution by either A or B red cells and causing the antibody solutions to agglutinate both A and B red cells.

The fact that the antibody solutions did not on any occasion agglutinate group O cells showed that the agglutination of heterologous cells, whether these were A or B, was not due to the presence in the group O sera of antibodies belonging to other blood groups systems.

*Series 2 : Experiments to prove that antibody solutions from group A cells prepared as in Series 1, in addition to anti-A contain anti-B, and those from group B cells, in addition to anti-B contain anti-A.*

These experiments involved neutralization of antibody solutions prepared as in Series 1 with the appropriate group-specific substance A or B. Thus, if the antibody had been recovered from group A cells, the anti-A activity of the solution was inhibited by the addition of group-specific substance A. The neutralised antibody solution was then tested against bloods of groups A and B. A similar experiment was made by neutralising the anti-B in an antibody solution prepared from B cells and testing this also against A and B bloods.

*Results.*—The results are shown in Table III, where it will be seen that an antibody which only agglutinated cells of group B was present in the solutions neutralized with group-specific substance A. This antibody can only be anti-B and therefore even though other antibodies in addition to anti-A might have been neutralized by the A substance (e.g. the hypothetical anti-C, see discussion) an explanation for the recovery of true anti-B from A cells has to be found. A similar argument applies to anti-A antibody recovered from B cells.

*Series* 3 : *Control experiments.*

(*a*) Antibody solutions prepared from packed washed group O cel's which had first been in contact with immune group O sera (Sparks case 4—Grayson case 2) were completely inactive, agglutinating neither A, B nor O cells.

(*b*) Antibody solutions obtained from A and B cells after treatment with immune sera of groups A and B did not cause any agglutination of heterologous red cells, i.e., antibody solutions from group B cells which had been treated with a serum containing only anti-A did not agglutinate cells of group A, while antibody solutions from group A cells which had been treated with a serum containing only anti-B did not agglutinate group B cells.

(*c*) Similarly when " artificial group O sera " were made by mixing equal parts of anti-A and anti-B immune sera, the antibody solutions prepared therefrom agglutinated either group A or group B cells but never both.

*Comment.*—The fact that group O cells are incapable of absorbing and releasing either anti-A or anti-B antibodies from group O sera refutes the possibility that the anti-A or anti-B recovered from heterologous cells in experiments in Series 1 is attached to those cells by means of a red cell receptor which is alike possessed by A, B and O cells. Thus the theory that linkage between anti-A and anti-B antibodies in immune group O sera exists is supported. Further confirmation for this linkage is afforded by the results of tests on antibody solutions prepared from monospecific sera containing one or other of the two antibodies, and by the results of experiments with " artificial " mixtures of anti-A and anti-B immune sera.

TABLE IV.—*Titres of Antibody Solutions Prepared from Non-immune and Random Group O Sera.*

| Cases. | Immune antibody. | Titre of original serum. | | | | Titre of antibody solution from A cells. | | | | Titre of antibody solution from B cells | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Anti-A. | | Anti-B. | | Anti-A. | | Anti-B. | | Anti-A. | | Anti-B. | |
| | | S | Co | S | Co | S | Co | S | Co | S | Co | S | Co |
| McRae pre-immunisation | — | 128 | 512 | 64 | 512 | 16 | 128 | 1 | 2 | 1 | 16 | 8 | 64 |
| Zeitlin pre-immunisation | — | 128 | 256 | 32 | 64 | 8 | 256 | — | 2 | 1 | 32 | 16 | 32 |
| Weir | — | 128 | 512 | 64 | 256 | 4 | 8 | — | 1 | — | 8 | 1 | 2 |
| Eeles | — | 256 | 8000 | 64 | 128 | 8 | 32 | — | 1 | — | — | 2 | 4 |
| 019 | — | 128 | 2000 | 8 | 8 | 4 | 128 | — | — | — | 64 | — | 1 |
| 097558 | — | 128 | 1000 | 32 | 256 | 2 | 8 | — | 4 | 4 | 32 | — | 8 |
| 44786 | — | 512 | 1000 | 128 | 256 | 16 | 64 | — | 2 | — | 2 | — | 16 |
| 46434 | — | 256 | 256 | 128 | 128 | 64 | 128 | — | 1 | 1 | 2 | 4 | 16 |
| 44982 | — | 128 | 128 | 64 | 128 | 64 | 64 | 2 | 4 | — | 1 | 32 | 32 |

In all cases the antibody activity of the final washings of both A and B cells was *nil.*

*Series* 4 : *Eluates made from A and B cells treated with non-immune and random group O sera.*

Four group O sera known to be non-immune and five random group O sera were investigated in the same way (Table IV). (It should be noted that in order to fulfil the definition " non-immune " for the purpose of this investigation, a serum must be from an individual who, so far as can be ascertained, has never (*a*) carried a foetus of group A or B, (*b*) received an incompatible blood transfusion

of group A or B blood, or (*c*) received an injection of substances containing antigens A or B.)

*Results.*—The titration results of antibody released from A and B red cells after treatment with these sera show a lower range of values than the immune sera, and only small amounts of anti-A are obtained in the antibody solutions from B cells and also of anti-B in the solutions from A cells.

*Comment.*—If a comparison of the titres of antibody in the solutions prepared from both immune and non-immune group O sera is made, it is found that those for anti-A or anti-B recovered from heterologous cells are very much higher than the corresponding ones for non-immune sera. Of particular interest are those cases in which experiments have been carried out on serum samples taken both before and after the injection of either A or B antigen. Therefore it seems that immune sera are a richer source of antibody units having two different combining sites than non-immune sera.

*Series 5 : Titration of group O sera after absorption with A and B red cells.*

The portions of group O sera which had been absorbed by A and B red cells for the preparation of antibody solutions by elution were kept and their anti-A and anti-B titres estimated.

*Results.*—These revealed that in many instances, accompanying the expected reduction in titre of anti-A and anti-B when each has been absorbed with homologous cells, there was also a reduction in the titre of the heterologous antibody. Examples of this are shown in Table V and indicated by an asterisk. The non-immune sera tested did not show this.

*Comment.*—Since it has been shown that anti-A antibody can be recovered from group B cells and similarly anti-B antibody from group A cells, a reflection of this in the titration values of the absorbed sera is to be expected. The reduction in titre of the heterologous antibody seems therefore to be due to the removal from the group O serum of that part of the heterologous antibody which is situated on the same globulin molecule as the homologous antibody. It will be noted that it is more markedly the titre of anti-B which reduced in sera absorbed by A cells. It is likely that a reduction of heterologous anti-A antibody does occur, but is not apparent owing to the limitations of the technique. This is probably because the anti-A titration values are considerably greater on the whole than those of anti-B, thus a significant change in titre is more difficult to obtain.

*Series 6 : Titration of group O sera after neutralization with A and B group specific substances.*

Seventeen of the same sera were titrated after neutralization with purified A and B group specific substances. The necessary amount of substance to neutralize approximately the homologous antibody was added to a small quantity of serum, and after a half to one hour the serum was titrated against A and B red cells. Experiments to control the specificity of the A and B substances were also included (see Table V).

*Results.*—These findings are very similar to those of Series 5. In point of fact the reduction in titre of the heterologous antibody is even more pronounced than when cells are used for absorption.

TABLE V.—*Titre of Immune and Non-immune Group O Sera after Absorption with A and B Group Specific Substances and A and B Red Cells.*

| Case. | Original serum titre. | | Titre after absorption with A substance. | | Titre after absorption with B substance. | | Titre after absorption with A cells. | | Titre after absorption with B cells. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Anti-A. S | Anti-B. S | Anti-A. S | Anti-B. S | Anti-A. S | Anti-B. S | Anti-A S | Anti-B. S | Anti-A. S | Anti-B. S |
| 3. Dark (immune anti-A) | 512 | 64 | – | 8* | 1000 | 2 | – | 4* | 512 | – |
| 5. Taylor ( „ „ ) | 4000 | 32 | – | 2* | 2000 | 1 | 2 | 4* | 4000 | – |
| 9. McRae ( „ „ ) | | | | | | | | | | |
| *a.* pre-immunisation | 128 | 64 | Not tested | Not tested | Not tested | Not tested | 2 | 32 | 128 | 1 |
| *b.* post-immunisation | 4000 | 1000 | 16 | 64* | 1000 | 1 | 8 | 64* | 4000 | 2 |
| 15. Zeitlin (immune anti-B) | | | | | | | | | | |
| *a.* pre-immunisation | 128 | 32 | Not tested | Not tested | Not tested | Not tested | 2 | 32 | 128 | 2 |
| *b.* Post-immunisation | 1000 | 4000 | 1 | 512* | 32* | 1 | 4 | 4000 | 128* | 8 |
| 20. Gore (immune anti-B) | 1000 | 2000 | 1 | 2000 | 8* | 2 | 4 | 2000 | 32* | 256 |
| 21. Sterile ( „ „ ) | 512 | 2000 | 8 | 2000 | 32* | 4 | 4 | 1000 | 64* | 2 |
| 44982 (non-immune) | 128 | 128 | 4 | 64 | 128 | – | 1 | 64 | 128 | 1 |
| 46434 „ | 256 | 128 | 1 | 64 | 256 | 1 | 1 | 64 | 128 | 1 |
| *Controls.* | | | | | | | | | | |
| Mixture anti-A + anti-B serum | 128 | 256 | – | 256 | 128 | – | | | | |
| Monospecific anti-A serum | 64 | – | – | — | 64 | – | | | | |
| Monospecific anti-B serum | – | 512 | – | 512 | – | – | | | | |

* Titre of heterologous antibody also reduced (see text, p. 9).

Results of the control experiments show that neither A nor B substance reduces the titre of the heterologous anti-B or anti-A antibody when these occur singly in group A and group B sera. Moreover if a mixture of anti-A and anti-B sera is made, there is no reduction in titre of the anti-A antibodies when group B substance is added, nor a reduction in titre of anti-B antibodies when group A substance is added.

*Comment.*—It is of particular interest that neutralization of the group O sera with A and B group-specific substances has a similar effect to absorption of the sera with A and B red cells, for since neutralization with group-specific substances does not remove antibody from the serum, as do red cells, the reduction in titre of the heterologous antibody cannot in this instance be due to its removal from the serum in combination with the homologous antibody. It seems extremely likely therefore that the suppression of activity of one or other antibody after the addition of the heterologous group specific substance is a spatial effect, and is brought about by the fact that the anti-A and anti-B combining sites of an antibody molecule which possesses both these specific sites are in close proximity. Thus the polysaccharide residues induce an " overshadowing " or steric hindrance of the heterologous receptors. Hence, a molecule of a particular group-specific substance is able effectively to screen a nearby combining group so that the latter is unable to combine specifically with its homologous determinate group. If this is true, absorption with red cells, or neutralization with group-specific substances of a serum from which the antibody molecules possessing two kinds of combining group have been removed, should not cause a reduction in titre of the heterologous antibody.

The following experiment was designed to test this point :

*Series 7 : Neutralization of a serum with group specific substance A after the serum had been absorbed with group A red cells.*

Serum McRae was selected for this experiment, and was first absorbed with packed washed group A red cells in the proportion of 2 ml. of serum to 10 drops of packed cells. A part of this absorbed serum was then subjected to a further neutralization with 0·25 ml. of group specific substance A. Anti-A and anti-B titrations were carried out by direct agglutination and Coombs' techniques both on the serum after absorption with A cells and after the further absorption with group-specific substance.

*Results.*—After absorption with A cells the anti-A titre was reduced from 4000 to 1 by direct agglutination methods and 4000 to 2 by Coombs' technique, and was accompanied by a reduction in titre of the heterologous anti-B agglutinin from 512 to 32 by direct agglutination method and from 2000 to 64 by Coombs' technique. The same serum sample after the addition of 0·25 ml. of A substance showed complete neutralization of anti-A agglutinin but no further reduction in titre of the anti-B agglutinin.

*Comment.*—The first absorption with A cells removed almost all the anti-A agglutinins, including those which were linked to anti-B. The removal of antibody molecules with both anti-A and anti-B combining groups is reflected in the considerable reduction in titre of anti-B. The further absorption with group A specific substance, while neutralizing the small amount of anti-A still remaining caused no further reduction in titre of the anti-B, presumably because all antibody

molecules possessing both anti-A and anti-B combining groups had already been removed from the serum.

*Series* 8*: Haemolysin experiments.*

Most of the immune sera lysed A cells and/or B cells to some extent. The non-immune sera caused slight lysis usually of only one kind of red cell. Eight of the immune sera lysing both A and B cells were titrated before and after absorption by A and B red cells.

*Results.*—The titres range from 2 to 16, but most are not higher than 4. Although every serum lysed both A and B cells, none lysed group O. Absorption of the sera by A and B cells produced an almost constantly different effect (Table VI). Thus if anti-A was the immune* antibody, absorption of the serum by A cells destroyed the lytic activity for both A and B cells, whereas absorption by B cells destroyed the anti-B lytic activity only.

TABLE VI.—*Haemolysin Titres of Immune Group O Sera Compared with those after Absorption with A and B Red Cells.*

| Cases. | Stimulating antigen. | Titre of haemolysin. Against A cells. | Against B cells. | Against O cells. | After absorption with A cells. Against A. | After absorption with A cells. Against B. | After absorption with B cells. Against A. | After absorption with B cells. Against B. |
|---|---|---|---|---|---|---|---|---|
| 2. Grayson | B | 8 | 16 | — | — | 4 | 2 | — |
| 3. Dark | A | 4 | 2 | — | — | — | 1 | — |
| 4. Sparkes | A | 16 | 4 | — | 1 | — | 16 | — |
| 9. McRae | A | 4 | 4 | — | — | — | 4 | — |
| 11. Pearson | A | 2 | 2 | — | — | — | 2 | — |
| 12. Young | B | 2 | 8 | — | — | 8 | — | — |
| 14. Tysson | B | 2 | 4 | — | — | 4 | 1 | — |
| 15. Zeitlin | B | 2 | 2 | — | — | 2 | — | — |

Conversely, if anti-B is the immune antibody, absorption with A cells removes all trace of anti-A haemolysin but the anti-B haemolysin remains while absorption with B cells leaves a serum which lyses neither A nor B cells.

*Comment.*—Increased lytic activity in immune sera is almost constant. It is also well known that immune group O sera, whether the anti-A or the anti-B is the immune agglutinin, will often lyse both A and B cells, although never group O cells. Hitherto this apparently non-specific lysis of heterologous cells has been difficult to explain, but these experiments suggest that they are lysed by antibodies which have both anti-A and anti-B combining sites. If the lysin is associated with the immune antibody and therefore with those immune antibodies which have both anti-A and anti-B combining sites, then it would be possible for a lysin associated with anti-A to lyse group B cells on account of close proximity with these cells, and conversely a lysin associated with anti-B to lyse group A cells for the same reason. Moreover this hypothesis would also account for the particular pattern of results obtained after the sera are absorbed with A and

* It should be explained that the term "immune" applies to that antibody in a group O serum which corresponds in specificity to the antigen which was responsible for the immunisation. The term "non-immune" is used to describe the antibody which is heterologous to the immunising antigen, e.g., if the immunising antigen is A (from red cells or group-specific substances) then anti-A is the immune antibody and anti-B is the non-immune antibody.

B red cells ; for if both anti-A and anti-B lysins are formed in the serum as entities which are separate from each other, then in all cases absorption with A cells should result in a serum which would haemolyse B cells only and *vice versa*. Alternatively, if a non-specific lysin had developed in the immune serum, absorption with either A or B cells should completely remove all lytic activity from the serum. It will be seen from a study of Table VI that neither of the two last possibilities fits the experimental findings.

## DISCUSSION.

Before discussing these results, it is necessary to define what they prove. They show that :

(1) Antibody solutions prepared from immune group O sera (Series 1) agglutinate both A and B red cells but not O cells.

(2) Antibodies responsible for this agglutination are in firm combination with the A and B red cells and only released on warming ; this is shown by the almost complete absence of antibody in the saline extracts obtained from the final washing of the agglutinated red cells before the warming takes place.

(3) Anti-B is present in antibody solutions prepared from group A cells, and anti-A is present in solutions prepared from group B cells (Series 2).

(4) Neither anti-A nor anti-B agglutinins are found in antibody solutions prepared from group O cells which have been in contact with immune group O sera (Series 3).

(5) It is not possible to recover heterologous anti-A or anti-B agglutinins from B or A cells respectively when these cells are allowed to absorb agglutinins from mono-specific anti-A (group B) or anti-B (group A) sera, or from mixtures of these agglutinins made by mixing together group A and group B sera (Series 3).

(6) Hence it can only be through the existence of antibody molecules in group O sera with combining groups of more than one specificity that the presence of anti-B in antibody solutions prepared from group A cells, or conversely anti-A in antibody solutions prepared from group B cells, can be explained satisfactorily.

(7) A comparison between results of experiments in Series 1 and 4 shows that immune group O sera contain more antibody molecules of the special type than non-immune group O sera. This is true irrespective of whether the immunisation is caused by a foetus *in utero*, incompatible red cells or injection of group-specific substances.

(8) The removal of heterologous antibody from an immune group O serum is often demonstrable by reduction in titre of that antibody after absorption of the serum with appropriate red cells (Series 5).

(9) When neutralization of either anti-A or anti-B agglutinins in group O immune sera by means of the homologous group-specific substances take place there is also a reduction in titre of the heterologous agglutinin (Series 6, Table V). If the specificity of the A and B substances is controlled (Table V) by testing them against anti-A and anti-B sera, both singly and as a mixture, no reduction in titre of heterologous antibody is detected.

(10) A group O serum which is absorbed with group A cells followed by treatment with group-specific substance A shows no further reduction in the titre of the anti-B after the addition of the group-specific substance.

(11) When the titre of *immune* antibody recovered from heterologous cells is compared with the titre of the non-immune antibody recovered from heterologous cells, a distinct pattern of results is formed. This is discussed later.

(12) Haemolysis of both A and B cells but not group O cells is caused by some immune group O sera.

(13) The haemolysin titres of these sera after absorption with A and B cells are shown to conform to a definite pattern as mentioned above.

So far the points enumerated have been borne out by experimental findings, and it is tempting to proceed further and suggest a theory to fit these findings. As mentioned previously, the presence of anti-A in antibody solutions prepared from group B cells and anti-B in antibody solutions from group A cells indicates

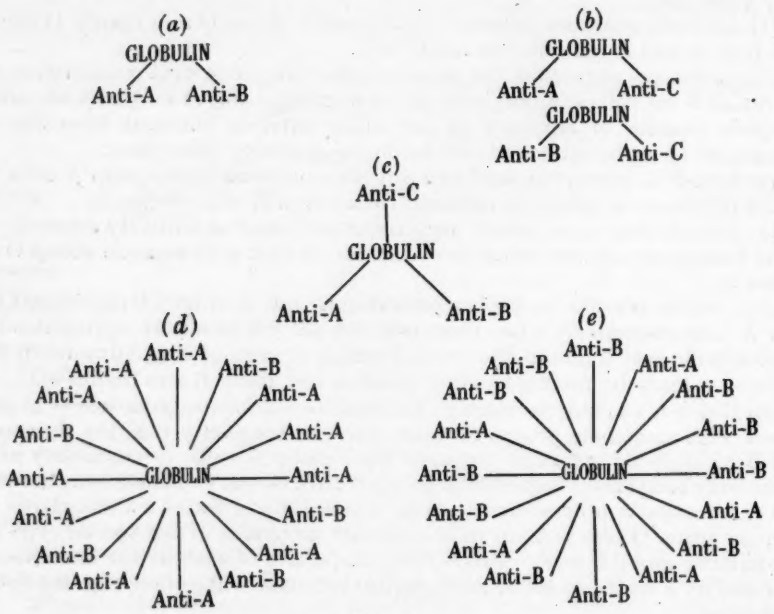


FIG. 1.

the existence of certain antibody molecules in group O sera which possess not only more than one combining receptor but receptors of different specificity. Fig. 1 (*a*), which is an attempt to depict this type of globulin molecule in a purely diagrammatic form, shows a bivalent structure with two receptors, one (anti-A) specific for antigen A and the other (anti-B) specific for antigen B. Thus a red cell of either group A or group B would be capable of taking up this type of antibody and afterwards releasing it on warming. Antibody solutions containing these antibodies would be capable of agglutinating both A and B red cells.

Although a direct linkage between anti-A and anti-B receptors or combining sites after this manner is most likely to be the correct explanation of the experi-

mental findings, the possibility of the existence of a third type of receptor (anti-C) in combination with anti-A and anti-B must be mentioned. The possible existence of a third type of antibody in group O sera was suggested by some early workers on blood groups, who postulated the presence of another antibody, anti-C, in addition to anti-A and anti-B, for which there was a specific antigen C which was alike possessed by A, B and AB red cells. The work of Hooker and Anderson (1921) seems to support this idea. They noticed that when certain anti-B immune rabbit sera were absorbed with group O blood, they still strongly agglutinated not only B cells but also A cells. Similar results were obtained by Landsteiner and Witt (1926) with immune anti-A sera.

However, the results of the absorption experiments of Koeckert (1920) and Schutze (1921) failed to indicate the presence of anti-C in group O sera, and most workers do not favour the existence of C and anti-C in the ABO blood-group system.

Diagrams introducing the hypothetical anti-C are shown in Fig. 1 (*b*) and (*c*). Fig. 1 (*b*) is a diagrammatic representation of globulin molecules of two kinds, one having combining sites anti-C and anti-A, and the other combining sites anti-B and anti-C. Fig. 1 (*c*) is an alternative to this arrangement in which anti-A, anti-B and anti-C combining sites are all situated on the same molecule. In either case A or B cells, each of which according to the theory possess antigen C, would be able to take up and release molecules of the types shown. Moreover antibody solutions which contained them would agglutinate both A and B red cells. Nevertheless the possible presence of this third antibody in group O sera, while difficult to disprove, does not remove the need for assuming the existence of molecules with combining sites of different specificities ; the anti-A which is recovered from B cells must be attached to anti-B or anti-C, and similarly the anti-B recovered from A cells must be attached either to anti-A or anti-C (see series 2) as the A receptors of red cells do not take up anti-B and *vice versa*. Thus if the choice lies, as it does, between anti-A and anti-B, each being the vehicle by which the other becomes attached to heterologous cells, or anti-C being responsible for this attachment, then the theory which need not include a hypothetical C and anti-C is much to be preferred. Experimental proof for a direct linkage between anti-A and anti-B might be forthcoming if blocking of the A or B receptors of red cells of groups A and B could be effected before these cells were allowed to absorb antibody from group O sera. Thus if the A receptors of group A cells could be saturated with anti-A antibody and then exposed to an immune group O serum, it might be possible to show that the antibody solution afterwards prepared only agglutinated cells of group A and not group B. This would show that blocking the A receptors of the cells so that they were no longer able to take up anti-A from the group O serum also prevented them from taking up and releasing anti-B. So it could be concluded that it is by means of linkage to anti-A that anti-B can be recovered from A cells. A similar experiment might be performed by blocking group B receptors with anti-B serum. Unfortunately preliminary experiments along these lines, although suggestive, have not been conclusive, owing to the technical difficulty of completely blocking the red cell receptors. Experiments in which the anti-A combining sites are blocked by neutralisation with group-specific substance A before the preparation of antibody solutions meet with greater success, for if this is done, anti-B is completely absent from anti-body solutions from A cells. However, it is conceivable that A and B group-specific substances might also contain the substance C, so for this reason

these experiments are not valid as a means of proving direct linkage between anti-A and anti-B.

A further question is whether the antibody molecules which have been shown to have at least two combining sites have in reality many more. An indication that the antibody molecules may indeed have more than two combining sites is gained from a comparison between the titres of immune and non-immune antibody recovered from heterologous cells.

Although in cases 8, 9, 10, 11, 14, 16 and 18 the titre of anti-A recovered from group B cells in each instance is approximately equal to the anti-B recovered from group A cells (Table II), in most of the remaining cases there is a definite, sometimes extremely marked difference between them, the titre of immune antibody recovered from heterologous cells being higher than the titre of non-immune antibody from heterologous cells. This is well shown by serum specimens 1, 4, 5, 7, 12 and 13, in which the immune antibody, anti-A, is present in unequivocally higher titre when recovered from group B cells than the non-immune anti-B recovered from A cells. In fact, in cases 1, 4 and 5 the titre of anti-A antibody recovered from group B cells far exceeds not only the titre of anti-B recovered from A cells but the original anti-B titre of the group O serum itself. Similarly, serum specimens 12, 15, 20, 21 and 22, in which the immune antibody is anti-B, conform to the same pattern. If anti-B is the vehicle by which anti-A is carried into the antibody solution prepared from B cells, it would seem to be impossible for the titre of anti-A from B cells to be greater than the original anti-B titre. It could be explained, however, by postulating multi-receptor antibodies (Fig. 1 (*d*) and (*e*)) with more immune than non-immune receptors. Fig. 1 (*d*) depicts the kind of antibody produced when A is the stimulating antigen, and Fig. 1 (*e*) the antibody produced to antigen B. Antibodies of the first type might give higher titration values with A cells, while antibodies of the second type might give higher titration values with B cells.

If the diagrams in Fig. 1 are representative of the kind of antibody molecules which exist in immune group O sera, it is easy to visualise the " overshadowing" or steric hindrance which would be caused when antibodies of this type, particularly those depicted in Fig. 1 (*d*) and (*e*), became attached to the relatively very large molecules of A or B group-specific substances which have been shown to have a particle size in excess of 250,000 (Aminoff, Morgan and Watkins, 1950). Here also it is interesting (Table V) that the non-immune antibody is much more readily overshadowed by its heterologous group-specific substance than the immune antibody. This finding supports the theory of multivalent antibodies of the type shown (Fig. 1 (*d*) and (*e*)). In Fig. 1 (*d*), for instance, the attachment between the anti-A receptors and molecules of group-specific substance A would readily cause overshadowing of the relatively few anti-B receptors, whereas an attachment between the anti-B receptors and molecules of group-specific substance B would cause less overshadowing of the larger number of anti-A receptors.

Whether a non-immune group O serum possesses antibodies with receptors or combining sites of two kinds is debatable, because it is impossible to be certain that the anti-A and anti-B agglutinins in a group O serum are non-immune. Substances containing antigens A and B or closely related substances are probably ubiquitous, and may easily gain access to the human body through the intake of food or by inhalation through the nasal mucous membrane. It is possible that droplets of saliva of group A, B or AB individuals which contain

potent group-specific substances are inhaled by group O individuals, resulting in at least a slight degree of immunization.

SUMMARY.

Antibody solutions prepared by warming either group A or group B red cells, after these cells have absorbed antibody from group O immune sera, are found to agglutinate both A and B red cells. This is due to the presence of both anti-A and anti-B antibodies in the solutions irrespective of whether these have been prepared from A or B cells.

These and other findings suggest that the immune group O sera tested contained some antibody molecules which possessed both anti-A and anti-B combining sites and which therefore could be taken up and subsequently released on warming by either A or B red cells. Solutions containing molecules of this type would be able to agglutinate both A and B cells.

Immune sera are a richer source of antibody molecules possessing both anti-A and anti-B combining sites than non-immune sera.

Neutralization of the anti-A antibody in immune group O sera by purified A group specific substance markedly reduced the titre of the heterologous antibody anti-B, and *vice versa*.

A series of haemolysin experiments demonstrated that immune O sera lysed both A and B cells whether the stimulating antigen was A or B. No lysis of group O cells took place. Non-immune sera showed very little lytic activity. The possibility that the haemolytic activity of antibody molecules possessing both anti-A and anti-B combining sites was responsible for the haemolysis of heterologous cells is suggested.

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# PORTAL HYPERTENSION IN THE RAT.

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ALTHOUGH portal hypertension is now a well-recognised clinical syndrome, attempts to reproduce the condition in animals by simple mechanical obstruction of the portal vein or its tributaries have so far been unsuccessful. It has often been shown that complete ligature of the portal vein in dogs, cats and rats is a fatal procedure (Elman and Cole, 1934; Boyce, Lampert and McFetridge, 1935; Menon, 1938), although Milnes and Child (1949) have shown that Rhesus monkeys will survive complete occlusion of the portal vein in one stage.

Warthin (1910) attempted to produce splenomegaly in rabbits and dogs by ligature of the splenic vein, but without success. Jäger (1931) constricted the portal vein of the dog and also did a reversed Eck fistula followed by ligation of the vein, but there was little splenic enlargement although histological changes were found in the spleen. Menon (1938) attempted partial occlusion of the portal vein in rabbits and rats by various techniques, but the results were somewhat inconstant although some rats showed splenic enlargement up to about twice the normal size. Rousselot and Thompson (1939) produced a condition resembling congestive splenomegaly with raised portal venous pressure in the dog by repeated injection of silica particles into the splenic vein, but this cannot be classified as a simple mechanical obstruction. Volwiler, Grindlay and Bollman (1950) were unable to produce a significant rise of portal venous pressure in the dog by various constrictions of the portal vein, although there was some rise after constriction of the intrathoracic inferior vena cava. There was no significant splenomegaly in these dogs.

The repeated failure to reproduce the human syndrome in a variety of experimental animals is unexplained, but most previous workers have been concerned primarily with splenic changes, and it was felt that a study of the effects of a sudden subtotal obstruction of the portal vein in the rat on portal venous pressure, the development of collateral circulation and the size of the spleen might provide some clue to the apparent paradox.

The effect of splenectomy on the portal venous pressure of an animal with portal hypertension would be worth investigating, as the operation is still performed on patients with Banti's syndrome with the object of reducing the portal venous pressure by cutting off some 20 per cent of the arterial inflow into the portal system.

## EXPERIMENTAL.

### *Constriction of the portal vein.*

Young female rats of the Wistar strain (weight 150–220 g.) were used. The portal vein was constricted by a modification of the method described by Whitaker

(1946). The animal was anaesthetized with ether and the duodenum was identified and withdrawn through a midline incision to expose the portal vein. A ligature was placed around the vein above the small pyloric vein, which is the last tributary received by the main trunk of the portal vein. A piece of wire 1/40 in. diameter was then placed alongside the vein and the ligature was tied tightly over it. The wire was then withdrawn and the abdomen closed in two layers. In a few animals a second ligature was tied loosely around the vein and the ends drawn out through the skin as described by Whitaker, to be pulled tight three days later, but this second ligature was of doubtful efficacy. About 50 per cent of the animals died within a few hours of operation, but the results of less severe constrictions were disappointing.

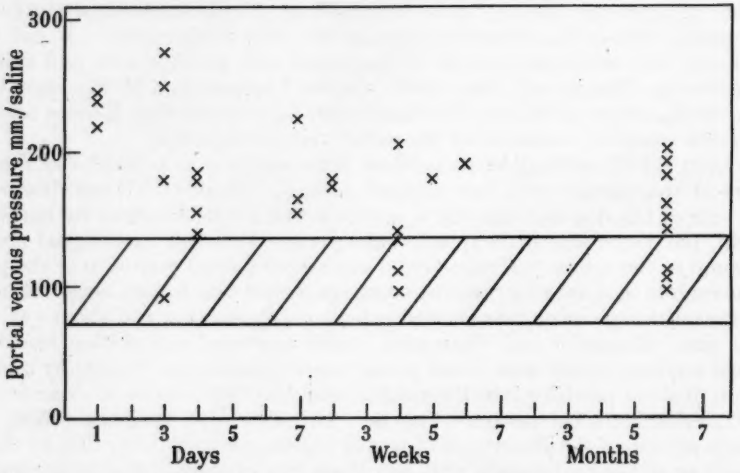



FIG. 1.—Readings of the portal venous pressure after constriction of the portal vein. The shaded area indicates the range of normal values.

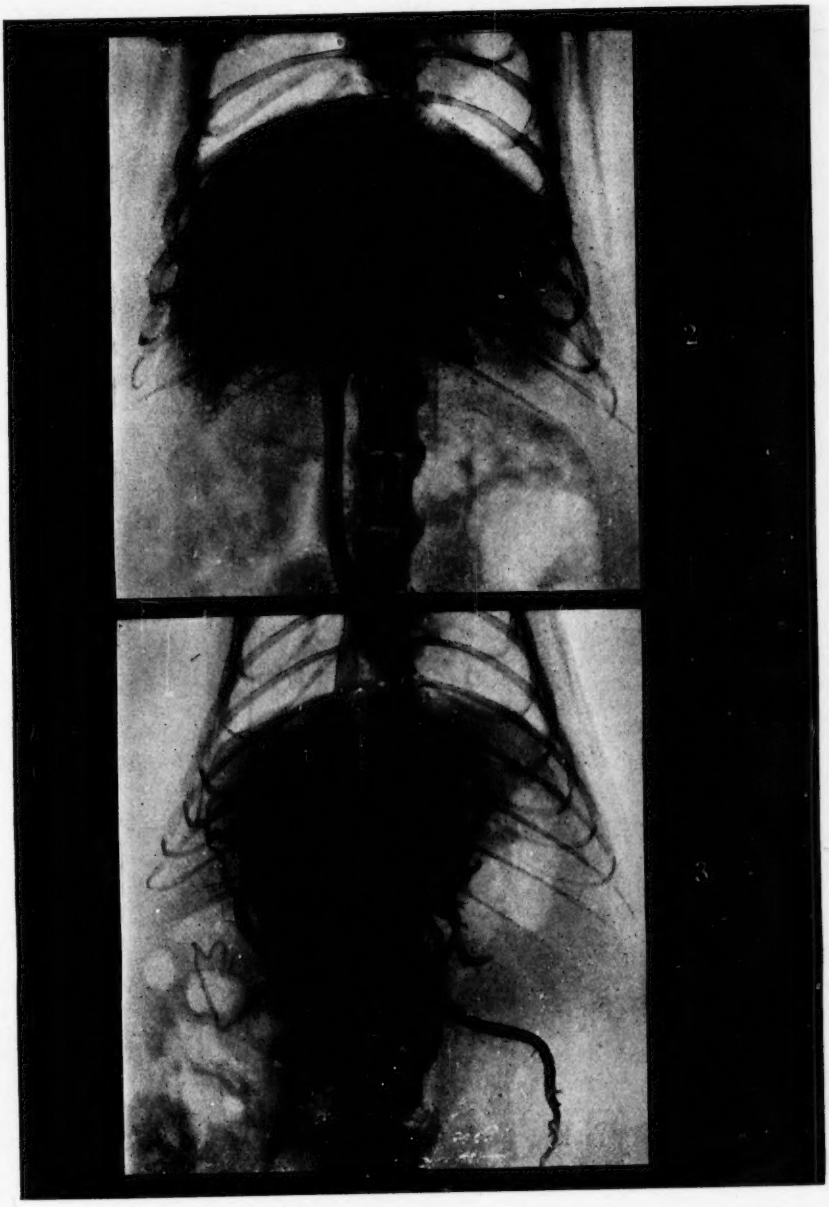
*The effect on portal venous pressure.*

The pressures were measured with a 20 gauge serum needle and a saline manometer. The needle was inserted about 2 cm. into the distal end of the superior mesenteric vein near the caecum and the column of fluid in the manometer was allowed to fall until a steady level was attained. Readings of the portal venous pressure were obtained in 24 normal rats of the same age, sex and strain as those subjected to constriction of the portal vein, and in these animals all values fell within the range 70–140 mm. saline. Pressures were also estimated in 35

DESCRIPTION OF PLATES.

FIG. 2.—Single frame from the angiogram of a normal living rat taken 3 sec. after the first appearance of contrast medium at the porta hepatis.

FIG. 3.—Single frame from the angiogram of a rat subjected to constriction of the portal vein 2 weeks previously, taken 3 sec. after the first appearance of contrast medium at the porta hepatis. Nearly all the portal venous blood is diverted away from the liver into collateral vessels (paraoesophageal, retroperitoneal and vessels to the anterior abdominal wall).



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animals at varying intervals of time after constriction of the portal vein and the results are shown in Fig. 1. In three animals the pressure was measured immediately after tying the ligature and values of 720, 470 and 380 mm. saline were obtained. Within 24 hours the pressure had fallen to the 200–270 range. By the end of the first week pressures varied between normal and twice normal, but thereafter showed no further tendency to fall ; even after six months pressures up to twice normal were recorded.

*The development of collateral circulation.*

The collateral circulation was studied in the living animal by the technique of rapid serial angiography which has been described more fully elsewhere (Daniel and Prichard, 1951). A small quantity of thorotrast was injected into a branch of the superior mesenteric vein of the anaesthetized animal, and the passage of the column of contrast medium through the liver and collateral vessels was recorded by serial radiographs taken at the rate of two per second. The collateral circulation was visualised in this way in eight animals which had been subjected to constriction of the portal vein one to six weeks previously (Fig. 2 and 3). In three of them this investigation was supplemented by the post-mortem injection of a suspension of bismuth carbonate into the superior mesenteric vein followed by stereoscopic radiography with the organs *in situ* and finally by dissection of the injected vessels.

It was apparent that the collateral circulation was fully formed by the end of the first week after operation, as angiograms taken after longer intervals did not suggest that collateral vessels had become either larger or more numerous. In all animals the portal vein and its tributaries were tortuous and distended and an extensive collateral circulation was always present. The anatomy of the collateral vessels bore some resemblance to those found after obstruction of the portal vein in man. The channels most frequently utilised were as follows :

1. Paraoesophageal vessels ran upwards to join the azygos or pulmonary veins. These were subserosal ; submucous oesophageal varices were not seen.
2. Retroperitoneal vessels anastomosed either with the inferior vena cava or the left renal vein.
3. Collateral vessels to the anterior abdominal wall formed at points at which the omentum became adherent to the parietal peritoneum.
4. Sometimes bloodflow in the inferior mesenteric vein was reversed, and blood escaped by way of anastomoses with the internal or common iliac veins.

*The effect on liver and spleen.*

Ten rats were subjected to constriction of the portal vein, and twelve others of the same age and sex were run concurrently as controls. At the end of six months all animals were sacrificed. The livers and spleens were excised and placed in a solution of normal saline. Within 90 minutes of excision they were mopped with filter paper and weighed. The weights are shown in Table I. Weights are expressed in mg. per 100 g. body weight, as Hatai (1913) has shown that the relationship between hepatic or splenic weight and body weight is approximately linear in Wistar rats of more than 140 g. The mean weight of the normal spleen was found to be 276 mg./100 g., which is almost identical with the figures given for the Wistar rat by Hatai (1913) and by Jackson (1915). Although the con-

striction appeared to have been ineffective in three of the rats (Nos. 4, 5 and 6), the mean weight of the liver was significantly less and that of the spleen significantly greater in the test group than in the controls. Further observations on splenic weights after constriction of the portal vein have been made during the course of other experiments and it has been found that the spleen is almost invariably significantly enlarged, but in no case did the splenic weight exceed 750 mg./100 g.

TABLE I.—*Weight of Liver and Spleen in mg./100 g. after Constriction of the Portal Vein.*

| Rat No. | Spleen weight. | Liver weight. |
|---|---|---|
| 1 | 401 | 3200 |
| 2 | 370 | 3650 |
| 3 | 378 | 3020 |
| 4 | 275 | 4000 |
| 5 | 378 | 4140 |
| 6 | 227 | 3870 |
| 7 | 400 | 3200 |
| 8 | 285 | 3100 |
| 9 | 308 | 3340 |
| 10 | 370 | 3780 |
| Mean | 339 | 3530 |
| Mean of 12 normals | 276 | 3840 |
| S.E. of difference | 22.6 | 139.5 |

*The effect of splenectomy.*

Sixty-six rats (56 female and 10 male) were subjected to constriction of the portal vein as already described. Those which survived the operation were left for one month and then subjected to a second laparotomy, at which the portal venous pressure was measured and haemostasis then secured with oxycel gauze. Any animal with a portal venous pressure of less than 130 mm. saline was

TABLE II.—*Portal Venous Pressures in mm. Saline Before and After Splenectomy and in Control Rats not Subjected to Splenectomy.*

| Splenectomy. | | | No splenectomy. | | |
|---|---|---|---|---|---|
| Rat. | Initial pressure. | Final pressure. | Rat. | Initial pressure. | Final pressure. |
| 1 | 160 | 165 | 1 | 165 | 135 |
| 2 | 185 | 170 | 2 | 150 | 175 |
| 3 | 170 | 170 | 3 | 140 | 130 |
| 4 | 180 | 170 | 4 | 150 | 130 |
| 5 | 150 | 135 | 5 | 130 | 155 |
| 6 | 160 | 180 | 6 | 180 | 165 |
| 7 | 180 | 205 | 7 | 135 | 180 |
| 8 | 130 | 200 | 8 | 200 | 165 |
| 9 | 170 | 210 | 9 | 195 | 165 |
| 10 | 165 | 160 | 10 | 195 | 165 |
| 11 | 190 | 150 | | | |
| Mean pressure change | + 8 | | | | —7 |

discarded. Of those with pressures greater than this, alternate rats were subjected to splenectomy. The animals were then allowed to recover and kept for a further month. At the end of this time the abdomen was again opened, a final reading of the portal venous pressure was made and all animals were sacrificed.

Splenectomy did not lead to any sustained fall in portal venous pressure, and there was no consistent change in pressure during the second month after constriction of the portal vein in either splenectomised animals or controls (Table II).

## DISCUSSION.

These experiments show that many of the features of portal hypertension in man can be reproduced by simple mechanical constriction of the portal vein in the rat. The difference between the two species is one of degree rather than one of kind. A sustained rise in portal venous pressure up to 200 mm. saline may be achieved in the rat, whereas in the human subject with portal vein obstruction it is usual to find pressures of 200–400 mm. saline (Milnes Walker, 1950). Similarly the splenic enlargement produced in the rat is significant, but not comparable in degree to that often found in man. The species difference is probably due to the facility with which collateral circulation develops in the rat. These studies of the portal venous pressure after constriction of the vein show that the operation results in an immediate rise in pressure, followed by a rapid fall within 24 hours and a slower fall during the next week. The fact that the collateral circulation appears to be fully developed by the end of the first week suggests that this fall in pressure is due to the rapid formation of collateral vessels. In the rat it seems that a portal venous pressure of more than about 250 mm. saline cannot be sustained for long, since at this level fresh collaterals will form rapidly, so that the outflow from the portal system is facilitated, and the portal venous pressure will fall to a level which is insufficient to stimulate the development of fresh collaterals. In man a greater difference between portal and systemic venous pressures is probably required before a significant collateral circulation will develop.

Splenectomy was first advocated in cases of Banti's syndrome by Banti himself (1898) in the belief that the disease had its origin in the spleen. It is now believed that in most if not all of these patients the primary cause of the syndrome is a raised portal venous pressure due to some mechanical obstruction of the portal or splenic vein (Whipple, 1945). It has nevertheless been argued that removal of the spleen will diminish the inflow of arterial blood into the portal system by about 20 per cent and thus reduce the portal venous pressure (McNee, 1932). This argument is a theoretical one, and there is no clinical or experimental evidence that removal of the spleen will in fact produce a sustained fall in portal venous pressure. It is obviously difficult to obtain this information in the human subject, but in rats with portal hypertension produced by subtotal constriction of the portal vein splenectomy did not lead to any sustained fall in portal venous pressure.

## SUMMARY.

The effects of a subtotal constriction of the portal vein of the rat have been studied.

The immediate rise of portal venous pressure produced by constriction of the vein is followed by a rapid fall, so that after a week pressures vary between normal and twice normal and thereafter there is no further fall.

An extensive collateral circulation resembling anatomically that found in the human subject is fully formed one week after constriction of the vein. There is a slight but significant reduction in the size of the liver and increase in the size of the spleen.

It is concluded that most of the features of portal hypertension in man can be reproduced in the rat by mechanical constriction of the portal vein. The difference is one of degree, and is apparently due to the greater facility with which collateral vessels develop in the rat.

Splenectomy did not lead to any sustained fall in portal venous pressure in these animals.

The radiological studies were made possible by the collaboration of Dr. P. M. Daniel and Miss M. M. L. Prichard.

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# A STUDY OF THE EFFECT OF CORTISONE IN EXPERIMENTAL SERUM DISEASE.

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IT has been shown by Rich and his co-workers (Berthrong, Rich and Griffith, 1950; Rich, Berthrong and Bennett, 1950) that administration of cortisone or ACTH modifies considerably the experimental cardiovascular and renal lesions produced by anaphylactic hypersensitivity. It seems important to attempt to confirm these observations since the results concerning the effectiveness of these new compounds in suppressing experimental hypersensitive reactions have been somewhat at variance, and since such a study might help towards a better understanding of the mechanism of their action in the so-called collagen diseases of man.

## EXPERIMENTAL.

The method of sensitisation was that originally used by Rich and Gregory (1943*a*, *b*) in their studies on the experimental production of periarteritis nodosa and serum carditis. Twenty rabbits were employed, a control group of ten which were treated with horse serum only, and a second group of ten which received cortisone in addition. The experimental procedure is outlined briefly in the Table.

TABLE.—*Experimental Procedure.*

| Rabbits. | 1st day. | 12th day. | 17th day. | 19th day. | 26th day. |
|---|---|---|---|---|---|
| 10 controls | 10 ml. horse serum I.V./kg. body weight | Skin test 0·1 ml. serum intracut. | 1 ml. serum I.V. | Initial dose of serum I.V. | Kill |
| 10 cortisone | 10 ml. horse serum I.V./kg. body weight and 7·5 mg. cortisone I.M. (daily). | Ditto | Ditto | Ditto | ,, |

The cortisone group received intramuscular injections daily of 7·5 mg. of this substance throughout the course of the experiment. Two large doses of intravenous horse serum were preferred, since in previous work (McKeown, 1947) this method has been found more successful in the production of experimental lesions. Skin testing was carried out on the 12th day, by intracutaneous injection of ·1 ml. horse serum. The animals were killed on the 26th day, and multiple sections were taken from all organs for histological examination.

## RESULTS

The number of animals used in this experiment was necessarily small, owing to restriction of supplies of cortisone, but the results in each group are so uniform that they may be regarded as significant. When skin-tested the cortisone group showed a complete suppression of skin reactivity. This finding is contrary to

the experience of Rich *et al.* (1950), but its importance is somewhat minimised by the fact that in six of the control animals there was only a slight reaction. Histological study revealed the presence of serum carditis in all the control animals, with involvement of the valves, valve angles, and coronary vessels in the manner fully described in previous reports. Peripheral vascular lesions were not frequent, six animals showing mild periarteritis in the spleen, and none the glomerulonephritis encountered by Rich and Gregory (1943*a*).

In the cortisone group there was a complete absence of vascular lesions in the heart and other viscera. This conclusion was arrived at only after histological examination of very many sections, especially of the heart. Each animal showed a high content of glycogen in the liver, and in five there was very pronounced extramedullary haemopoiesis in the spleen. Renal changes were not conspicuous in the cortisone group, but in one animal there were a few fibrin thrombi in the glomeruli and blood and casts in the tubules. In two animals the lining epithelium of some of the tubules was vacuolated, resembling the Armani Ebstein cells found in diabetes. No significant histological changes were detected in the adrenals of this group of animals, though there was possibly some increase of lipoid in the zona fasciculata.

## DISCUSSION.

In spite of the small number of animals used in this experiment the results in each group are quite consistent, and it seems reasonable to conclude that cortisone exerts a suppressive action upon the experimental cardiovascular lesions produced by anaphylactic hypersensitivity. The real problem lies in determining at what point in the hypersensitive reaction cortisone exerts this effect. Theoretically it might do so in one of three ways, by interfering with the production of antibody, by preventing the inter-reaction of antigen and antibody, or by altering the susceptibility of the tissues to injury.

The experiments described here do not serve to distinguish between these three possibilities. Published evidence does not support the first two, and if the third be accepted, the elucidation of its precise mechanism is a matter of wide interest, since it now seems possible to prevent certain disease processes, not by inactivating their cause, but by so altering the state of the tissues that they are protected from injury.

## SUMMARY.

Twenty rabbits were sensitized with horse serum in the manner known to produce serum carditis and periarteritis nodosa. Ten received daily injections of cortisone throughout the course of the experiment. Skin reactivity was suppressed in the cortisone-treated group, and there was a complete absence of lesions of the cardiovascular system. The results suggest that cortisone has a marked inhibitory effect on the development of the cardiovascular lesions of hypersensitivity.

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# THE NUTRITION OF *PASTEURELLA SEPTICA*. I. THE ACTION OF HAEMATIN.

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It has long been known that some strains of *Pasteurella pestis* and *Pasteurella septica* fail to grow when small numbers of organisms are seeded on to certain media which will support the growth of large inocula. A reducing agent, sodium sulphite, was found to allow the growth of *Past. pestis* from small inocula (Drennan and Teague, 1917; Meyer and Batchelder, 1926) and Webster (1925) observed that the growth of *Past. septica* was promoted by a low oxygen tension or by a heat-stable factor in blood. The latter could be replaced by certain inorganic compounds of iron with peroxidase-like properties (Webster and Baudisch, 1925). Schütze and Hassenein (1929), using *Past. pestis*, found that the blood factor could also be replaced by a heat-labile factor present in filtrates of cultures of Pasteurella species and of a wide variety of other organisms, of which the greater number were catalase producers. Herbert (1949) identified the growth-promoting component of blood as haematin and found that it could be replaced by certain reducing agents or by blood charcoal, which catalytically decomposes hydrogen peroxide. He suggested that under aerobic conditions hydrogen peroxide was formed and inhibited growth unless its accumulation was prevented. Haematin was assumed to act by promoting the synthesis of catalase by the organism.

Some of these findings have been confirmed with a strain of *Past. septica* in experiments described in this paper and the mode of action of haematin investigated further.

## METHODS.

### *Organism used.*

This was a virulent mouse strain obtained from Prof. J. C. Cruickshank. It was preserved in the dried state by the method of Stamp (1947). Dried cultures were opened at monthly intervals and grown on blood-agar slopes. These were stored at 0° and used to seed similar slopes for current use. Inocula for growth tests were prepared from the 18-hr. growth on these slopes by suspending the cells in M/30 phosphate buffer pH 7·6 and diluting to the required concentration. Liquid cultures were usually inoculated with $10^3$ cells/ml.

### *Media.*

*Tryptic digest of beef* (double strength) was made by the method of Douglas modified as described by Fildes and McIntosh (1931). It was diluted for use with an equal volume of distilled water and autoclaved at pH 7·6. The final product (TMB) contained 3·5 to 4 per cent soluble solids and 0·65 to 0·75 mg./ml. amino N.

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*Tryptic meat agar* (TMA) was made by adding sterile double strength beef digest to an equal volume of sterile 3 per cent Bacto-agar (Difco Laboratories) which had been autoclaved in M/300 phosphate buffer, pH 7·6.

*Other compounds* tested were as follows: *blood*—human blood (containing 0·44 per cent disodium citrate) or horse blood (oxalated, Burroughs Wellcome & Co.); *peptic digest of sheep's blood*—prepared by the method of Fildes (1920); *catalase*—from blood (a sample of the enzyme kindly given by Dr. D. Herbert), sterilized by filtration through a Chamberland candle filter; *haematin*—a crystalline sample of haemin prepared as described by Gattermann (1937), dissolved in dilute NaOH and autoclaved; *thiolacetic acid*—British Drug Houses spot test reagent, neutralized and Seitz filtered; *sodium sulphite* and *manganese dioxide*, Harrington Bros., autoclaved.

*Conditions of incubation.*

Stationary cultures were usually incubated in 10 ml. volumes in 50 ml. Erlenmeyer flasks in air. To aerate cultures, air was bubbled through the medium through sintered glass inlet tubes. Anaerobic cultures were incubated in McIntosh and Fildes jars filled with hydrogen/5 per cent $CO_2$. All cultures were incubated at 37°.

*Measurement of growth.*

*Total counts* were estimated from the turbidity of liquid cultures measured photoelectrically.

*Viable counts* on different media were compared by the surface counting method of Miles and Misra (1938). Plates were incubated for 2 days. Viable counts in liquid media were determined by the roll-tube method of Wilson (1922).

*Chemical methods.*

*Haematin* was estimated by the pyridine-haemochromogen method of Rimington (1942).

*Catalase activity* was determined by the method of Euler and Josephson as described by Herbert and Pinsent (1948). It is expressed as $k_o$, the first order velocity constant at zero time for the decomposition of hydrogen peroxide. Estimations were made with intact cells, as the results obtained were not increased when bacteria had been disintegrated by shaking with glass beads.

*Hydrogen peroxide* was detected by its oxidation of phenolphthalin to phenolphthalein in the presence of $Cu^{\cdot\cdot}$ (Schales, 1938). A tenfold increase in the concentration of $Cu^{\cdot\cdot}$ was required when testing for hydrogen peroxide in the presence of bacteria.

## RESULTS.

### *The Requirement for Haematin for Growth.*

*The effect of blood and haematin on growth on tryptic meat agar.*

This organism grew on tryptic meat agar (TMA) forming colonies varying from 1 to 2 mm. in diameter in 48 hours. When, however, viable counts were done on this medium, the numbers of colonies developing from inocula of constant size, as shown by turbidity measurements, were very variable. For example, the average counts in four successive experiments were 274, 195, 1 and 29.

The addition of 5 per cent blood reduced the discrepancies between counts to values within the experimental error. Moreover, the rate of growth and size of colonies were considerably increased, colonies of 2½ to 3 mm. diameter being obtained in 24 hours. The same result was obtained when blood was replaced by a peptic digest of blood. The effect of adding a concentration of haematin (50 μg./ml.) equivalent to that added in the blood digest was therefore tested. Haematin had no effect on the size of colonies growing on TMA and only slightly increased the rate of growth. However, colony counts on media to which it had been added did not differ significantly from those on blood and peptic blood plates (Table I). A comparison of sub-optimal concentrations of the blood digest and

TABLE I.—*The Effect of Blood and Haematin on Counts on Tryptic Meat Agar.*

| Experiment. | Additions to TMA. | Equivalent haematin concentration. | Number of colonies/plate. |
|---|---|---|---|
| I | *nil* | .. | 49 |
| | Horse blood 5 per cent | 225 μg./ml. | 301 |
| | Peptic sheep's blood 5 per cent | 55 „ | 292 |
| | Haematin 50 μg./ml. | .. | 286 |
| II | *nil* | .. | 32 |
| | Peptic blood digest 5 per cent | 55 μg./ml. | 130 |
| | „ „ „ 0·5 „ | 5 „ | 85 |
| | Haematin 50 μg./ml. | .. | 143 |
| | „ 5 „ | .. | 77 |

Each result is the mean of the count on 4 plates.

of haematin indicated that the effect of the former on colony counts was entirely dependent on its haematin content.

*The effect of catalase on growth on tryptic meat agar.*

As it has been suggested that haematin is utilised by bacteria for the synthesis of catalase, the effect of catalase on growth was tested. It was found to be 50 times more effective than haematin, 1 μg. replacing 50 μg. of the latter. Its activity was reduced to that of its haematin content by boiling (Table II).

TABLE II.—*The Effect of Catalase on Colony Counts on Tryptic Meat Agar.*

| Experiment. | Additions to TMA. | Number of colonies/plate. |
|---|---|---|
| I | *nil* | 36 |
| | Catalase 1 μg./ml. | 657 |
| | „ 0·1 „ | 383 |
| | Haematin 50 „ | 625 |
| | „ 5 „ | 355 |
| II | *nil* | 65 |
| | Catalase 1 μg./ml. | 178 |
| | „ 1 „ after 5 min. at 100° (= 0·5 μg. haematin/ml.) | 115 |
| | Haematin 50 μg./ml. | 167 |
| | „ 0·5 „ | 109 |

Results in Exp. 1 are the mean values obtained on 4 plates each, and in Exp. II on 3 plates each.

*The effect of oxygen tension on growth.*

Since catalase catalyses the decomposition of hydrogen peroxide (a product of aerobic metabolism) tests were made to investigate whether haematin was required for anaerobic growth and growth at a lowered oxygen tension. A reduction in oxygen tension was obtained by covering plates with a layer of agar after inoculation. Table III shows that under anaerobic conditions, counts in the

TABLE III.—*The Effect of Oxygen Tension on Colony Counts on Tryptic Meat Agar.*

| Additions to TMA. | Conditions of incubation. | Number of colonies/plate. |
|---|---|---|
| *nil* | Aerobic | 364 |
| *nil* | ,, (plate covered by 10 ml. TMA) | 550 |
| *nil* | ,, ,, ,, 20 ,, | 651 |
| *nil* | Anaerobic | 822 |
| Haematin 50 μg./ml. | Aerobic | 828 |

Each result is the mean of counts on 2 plates.

absence of haematin did not differ significantly from those obtained aerobically on haematin-containing media, but that at a low oxygen tension counts were intermediate between the aerobic and anaerobic values. The differing sensitivity of individual cells to air was comparable with their wide variation in haematin requirements, as shown by the gradual reduction in counts with decreasing haematin concentrations (Tables I and II).

The relation between the effects of haematin and of oxygen tension could also be demonstrated by incubating cultures in test-tubes in semi-solid media made by the addition of 0·1 per cent agar to reduce the rate of diffusion of air. Growth first appeared about 1 cm. below the surface and gradually extended upwards and downwards. The addition of haematin or a reducing agent (thiolacetic acid) decreased the time required for growth to reach the surface.

*Growth in tryptic meat broth.*

Visible growth always occurred in tryptic meat broth (TMB) even from small inocula ($10^2$ cells/ml.). Haematin had little effect in stationary cultures. It significantly increased the rate although not the amount of growth in aerated cultures. An inoculum of $10^3$ cells/ml. grew in TMB to $2 \times 10^7$ cells/ml. in 9 hours and reached a maximum in 16 hours, whereas with 50 μg. haematin/ml. a similar inoculum grew to $2{\cdot}3 \times 10^8$ cells/ml. in 9 hours and had reached a maximum in 11 hours.

*Serial subculture in the absence of haematin.*

The growth of a small part of each inoculum in the absence of haematin suggested that it might be possible to select out a non-haematin requiring strain. However, on subculturing organisms in TMB and subsequently determining the proportion of haematin-requiring cells by a comparison of counts on TMA with and without haematin, no significant change as compared with cultures in TMB —haematin was found after as many as 17 serial transfers. These results also showed that the growth of a small part of the inoculum was not due to the transfer

of haematin from the preceding culture fluid, otherwise continued subculture in the absence of haematin should have depleted cells sufficiently to inhibit growth completely.

*Haematin requirements in a chemically-defined medium.*

Although meat is rich in haematin compounds, it is probable that they are removed by adsorption on to the phosphate precipitate filtered off during the preparation of the meat digest (Herbert, 1949). As it was possible that sufficient haematin remained in the medium to permit the growth of a few colonies, its effect was determined in a chemically-defined medium. In such a medium, as described in a subsequent paper (Jordan, 1952), the results obtained in meat media were confirmed, some growth always occurring both in solid and liquid media in the absence of haematin.

*The replacement of haematin by other compounds.*

As catalase would replace haematin for growth on TMA, a number of other compounds which also promote the decomposition of hydrogen peroxide were tested. Results in Table IV show that sodium sulphite, sodium thiolacetate sodium pyruvate and manganese dioxide were all able to replace haematin in TMA.

TABLE IV.—*The Replacement of Haematin by other Compounds on Tryptic Meat Agar.*

| Experiment. | Additions to TMA | Number of colonies/plate. |
|---|---|---|
| I | *nil* | 88 |
| | Na pyruvate M/100 | 468 |
| | Na thiolacetate M/100 | 386 |
| | Na sulphite M/250 | 368 |
| | Haematin 50 $\mu$g./ml. | 454 |
| II | *nil* | 15 |
| | $MnO_2$ 1 per cent | 131 |
| | Haematin 50 $\mu$g./ml. | 149 |

Each result is the mean of counts on 2 plates.

*The utilization of haematin during growth.*

No detectable quantity of haematin (i.e., $<2\mu$g./ml.) disappeared from the medium during growth. Although very small amounts of haematin might have a marked effect on the cell content of haemoproteins, it was of interest that there was such a big discrepancy between the optimum requirement for growth and the amount utilized. A comparison of the catalytic activity of 50 $\mu$g. haematin with that of an 18-hour blood slope culture as normally used for the inoculation of growth tests showed that the activity of this quantity was of the same order of that of $10^7$ cells. Its catalytic activity might thus be of some importance in the protection of isolated cells seeded on plates or inoculated into liquid media at the low concentration ($10^3$ cells/ml.) used.

*The effect of haematin on catalase formation during growth.*

It was apparent that the effect of haematin could only partially be accounted for by its catalysis of the decomposition of hydrogen peroxide in the medium, as catalase which is about 2 million times as active as haematin catalytically was only about 20 thousand times as active in promoting growth. It seemed possible, therefore, that haematin might also act, indirectly, by stimulating catalase production by the bacteria. It was found, however, that haematin had only a slight effect on catalase formation. Table V shows typical results. Cultures were

TABLE V.—*The Effect of Haematin on Catalase Formation.*

| | Catalase activity/g. bacterial dry wt. ($k_0$). | | | |
|---|---|---|---|---|
| | Aerated cultures. | | Stationary cultures. | |
| Time (hr.). | No haematin. | Haematin (50 μg./ml.). | No haematin. | Haematin (50 μg./ml.). |
| 2 | 18 | 18 | 11 | 10 |
| 5 | 21 | 20 | 12 | 13 |
| 8 | 19 | 18 | 16 | 14 |
| 11 | 21 | 50 | 10 | 9 |
| 22 | 7 | 7 | 6 | 10 |

Basal medium: TMB. Inoculum: $2 \times 10^7$ cells/ml. of a washed suspension prepared from a 24-hr. aerated culture in TMB-haematin. Catalase activity of inoculum = 7.

seeded with large inocula so that sufficient organisms could be obtained for the determination of catalase activities early in growth. In stationary cultures haematin had no detectable effect. When cultures were aerated, the catalase content of cultures both with and without haematin rapidly increased but the maximum obtained was greater with haematin. The catalase activity of cultures always dropped rapidly at the end of growth.

These results show that some increase in catalase content took place during the first two hours of incubation. In other experiments it was found that there was no detectable change during the lag phase, which occupied about one hour.

## *The Inhibition of Growth by Hydrogen Peroxide.*

*The effect of added hydrogen peroxide on growth.*

As haematin was only required for optimum growth under aerobic conditions and could be replaced by substances which decomposed hydrogen peroxide, it was of interest to determine whether this organism was particularly sensitive to hydrogen peroxide. The toxicity of hydrogen peroxide was the same in TMB as on TMA, complete inhibition of growth being caused by 10 μg./ml. in the absence of haematin. It is difficult to compare this figure with other observations because of the effect of different basal media, but some idea of the sensitivity of this organism compared with others may be obtained from a survey by McLeod and Gordon (1923) of a large number of organisms grown on infusion agar. They found that concentrations of hydrogen peroxide ranging from 17 to 150 μg./ml. were required to inhibit the growth of all aerobic strains tested, whether catalase producers or not, except *Vibrio cholerae*, which was inhibited by 7 μg./ml.

Results in Table VI show that the addition of haematin reduced the toxicity of hydrogen peroxide in TMA at least 100 times. As haematin decomposes

TABLE VI.—*The Effect of Hydrogen Peroxide on Growth.*

| Basal medium. | Hydrogen peroxide ($\mu$g./ml.). | Number of colonies/plate. |
|---|---|---|
| TMA | 0 | 133 |
| | 4 | 114 |
| | 20 | 0 |
| TMA-haematin 50 $\mu$g./ml. | 0 | 178 |
| | 20 | 217 |
| | 100 | 194 |
| | 500 | 0 |

Each result is the mean of counts on 2 plates.

hydrogen peroxide slowly and is itself oxidised by it, results in the presence of haematin are dependent on the combined effects of haematin and hydrogen peroxide both at concentrations lower than the stated values. In a chemically-defined medium containing haematin (Jordan, 1952) hydrogen peroxide was found to be more than 100 times as toxic as in TMA-haematin medium.

*The formation of hydrogen peroxide by the organism.*

A trace of hydrogen peroxide (about 0·1 $\mu$g./ml.) was present in TMB, but there was no detectable quantity (i.e., $<0{\cdot}01$ $\mu$g./ml.) in the chemically-defined medium. To account for the importance, for aerobic growth, of compounds decomposing hydrogen peroxide it was therefore necessary to assume that this bacterium formed hydrogen peroxide as a product of its own metabolism. It seemed improbable that hydrogen peroxide would be detectable in growing cultures, because growth itself (and therefore $H_2O_2$ production) would depend upon the peroxide not accumulating above the toxic concentration. None was in fact found, either by culture on benzidine-blood-agar (Penfold, 1922), on " chocolate-" agar (McLeod and Gordon, 1922 ; Hart and Anderson, 1933) or on media containing manganese dioxide (Kneteman, 1947). Schönbein's reagent (McLeod and Gordon, 1922) and phenolphthalein (Schales, 1938) gave negative results when used to test samples of liquid media removed during growth.

The formation of hydrogen peroxide could be demonstrated during the oxidation of a number of substrates by a washed suspension of cells shaken in air at 37° in Warburg constant volume manometers. Approximately 1 mg. (dry weight) of cells was used in 3 ml. of fluid buffered at pH 7·6 with M/90 phosphate. When 1 ml. TMB was added as substrate there was no increase in the hydrogen peroxide concentration over the initial value during respiration. However, when M/1000 hydroxylamine was added to inhibit the catalase activity of the suspension, between 0·5 and 1$\mu$g. peroxide/ml. could be demonstrated with the phenolphthalein reagent at the end of one hour's shaking, although the rate of oxygen uptake had been decreased by about 80 per cent. This reagent is not completely specific, but the probability that the oxidising agent formed was hydrogen peroxide was confirmed by the prevention of the reaction by the addition of catalase prior to the addition of the reagents. Traces of hydrogen peroxide (0·01 to 0·05

$\mu$g./ml.) could also be detected when cell suspensions were shaken with the amino-acid mixture used in chemically-defined media, if hydroxylamine was also added.

## DISCUSSION.

These results are in agreement with the observations of previous workers that haematin is required for the aerobic but not the anaerobic growth of this organism. Furthermore, the supposition that haematin is required to prevent the inhibitory effects of hydrogen peroxide was confirmed by the observations that haematin considerably reduced the toxicity of added hydrogen peroxide, and that hydrogen peroxide was formed during the oxidation of certain metabolites.

The mechanism by which haematin protects this organism, which is itself catalase-producing, is not clear, as its catalysis of hydrogen peroxide decomposition cannot account for all its activity. Herbert (1949) suggested that the cell catalase content might be inadequate during the early stages of growth unless haematin was present to promote synthesis. However, no evidence could be obtained that haematin promoted catalase synthesis at the beginning of growth. Indeed, in stationary culture, when growth was limited by the supply of air, haematin had no detectable effect on catalase formation.

As peroxidase has a much higher affinity for hydrogen peroxide than catalase it might be of considerable importance in the removal of small quantities of peroxide. Preliminary experiments using benzidine as a reagent indicated that about one-third of the peroxidase activity of this organism was heat-labile and therefore probably enzymic. There was no activity detectable by this method in cells grown in a medium lacking haematin. No definite conclusion can be drawn from experiments based on an unspecific reagent such as benzidine, but these results suggest another line of attack on the problem.

In common with most facultative anaerobes, this organism contains components of the cytochrome system. A typical spectrum can be observed in cells grown in TMB with or without haematin. It was not determined whether growth in haematin increased the cytochrome content, as the added haematin interfered with observations under the conditions used. It was noted, however, that growth in haematin had a marked effect on the respiratory activity of washed cell suspensions. Whereas it increased the rate of oxygen uptake in the presence of succinic acid, it depressed it in the presence of TMB, amino-acids and glucose. Results presented in a subsequent paper (Jordan, 1952) show that the oxidation of succinic acid but not of glucose was prevented by inhibitors of the cytochrome system. The oxidation of glucose like that of TMB and amino-acids was associated with the production of hydrogen peroxide. Further investigations may therefore indicate that part of the protective effect of haematin is mediated by an increase in metabolism through the cytochrome system and a decrease in the rate of peroxide-forming oxidations.

## SUMMARY.

A strain of *Pasteurella septica*, which requires blood for its optimum aerobic growth on nutrient agar, will also grow readily when blood is replaced by haematin, catalase, or a number of compounds which are able to catalyse the decomposition of hydrogen peroxide. Blood and the compounds which will replace it are not required for anaerobic growth. Evidence is presented that they prevent the

inhibition of growth in air by protecting the organism from the toxic effects of hydrogen peroxide formed as a product of its own metabolism.

Haematin catalyses the slow decomposition of hydrogen peroxide. It promotes the formation of catalase in aerated cultures, but in stationary cultures haematin has no effect on the production of this enzyme. These observations are not sufficient to account for the effect of haematin on growth, and some other possible mechanisms are discussed.

I am greatly indebted to Dr. G. P. Gladstone, under whose supervision this work was done, for helpful advice and criticism. My thanks are also due to Prof. D. Keilin and to Dr. D. Herbert for useful suggestions. I also wish to thank the Medical Research Council for a Studentship.

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# THE NUTRITION OF *PASTEURELLA SEPTICA*. II. THE FORMATION OF HYDROGEN PEROXIDE IN A CHEMICALLY-DEFINED MEDIUM.

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ALTHOUGH the growth requirements of *Pasteurella pestis* are well defined, very little is known about the nutrition of *Past. septica*. Koser, Berkman and Dorfman (1941) found that a number of strains would grow on a glucose-hydrolysed gelatin medium supplemented with amino-acids if nicotinamide and pantothenic acid were added, but failed to obtain growth in a chemically-defined amino-acid mixture (Berkman, 1942).

During the determination of the growth requirements of a virulent strain of *Past. septica* it was observed that inhibitory quantities of hydrogen peroxide were formed in the presence of certain sugars. This has now been investigated in detail and the findings described in this report. Haematin is required for the optimum aerobic growth of this strain as for many other strains of *Past. septica* and *pestis*. This has already been discussed (Jordan, 1952), and all growth experiments described here have been carried out in media containing 50 $\mu$g. haematin/ml.

## METHODS.

The organism used, its routine culture for the preparation of inocula, the measurement of cell growth and the preparation of tryptic meat broth and agar (TMB and TMA) have already been described (Jordan, 1952). The conditions of incubation were also as described previously except that cultures in chemically-defined media were incubated in air/5 per cent $CO_2$. Viable counts on plates prepared from chemically-defined media were compared after 4 days' incubation with counts on TMA-haematin.

### *Chemically-defined media.*

The initial basal medium used is given in List I and was later modified in accordance with experimental findings (List II). It may be noted that nicotinamide, pantothenic acid and aneurin were found to be essential. Oleic acid increased both the rate and amount of growth, the optimum concentration being 10 $\mu$g./ml. Cystine was the only essential amino-acid and was required at a high concentration. Final concentrations are given below, the quantities used being convenient for rapid weighing. Sugars and lactic and pyruvic acids after neutralization were Seitz filtered and added to the medium separately. The water used throughout was glass distilled. The chemicals used were as follows:

* Medical Research Council Student.

sucrose, glucose and lactic acid—British Drug Houses "Analar"; fructose, inorganic salts and citric acid—Harrington Bros.; oleic acid—Hopkins & Williams "purified," twice distilled. All other compounds were British Drug Houses laboratory reagents, except glucose-l-phosphate, which was kindly given by Dr. C. S. Hanes.

*Growth factors.*

| | List I. | List II. | |
|---|---|---|---|
| Adenylic acid | M/20,000 | Omitted | Dissolved together in water and Seitz filtered. |
| Cytosine | M/40,000 | ,, | |
| Guanine | ,, | ,, | |
| Thymine | ,, | ,, | |
| Uracil | ,, | ,, | |
| Aneurin | M/100,000 | As in List I | |
| Nicotinamide | M/50,000 | ,, | |
| Ca pantothenate | ,, | ,, | |
| *p*-Aminobenzoic acid | M/500,000 | Omitted | |
| Biotin | 0·001 μg./ml. | ,, | |
| Folic acid | 1·0 μg./ml. | ,, | |
| Pyridoxine | M/500,000 | ,, | |
| Riboflavin | ,, | ,, | |
| Haematin | 50 μg./ml. | As in List I | Dissolved in N/100 NaOH and autoclaved. |
| Oleic acid | 10 μg./ml. | ,, | Neutralized and autoclaved. |

*Amino-acids.*

| | List I. | List II. | |
|---|---|---|---|
| Glycine | M/750 | As in List I | Dissolved together in M/30 phospate buffer and autoclaved |
| DL-Alanine | M/890 | ,, | |
| DL-Valine | M/780 | ,, | |
| DL-Leucine | M/875 | ,, | |
| DL-Isoleucine | M/1,310 | ,, | |
| DL-Aspartic acid | M/665 | ,, | |
| DL-Glutamic acid | M/735 | ,, | |
| L-Proline | M/2,300 | M/575 | |
| L-Hydroxyproline | M/2,620 | Omitted | |
| DL-Phenylalanine | M/1,650 | As in List I | |
| DL-Tyrosine | M/1,810 | ,, | |
| L-Arginine | M/4,200 | ,, | |
| L-Lysine | M/3,640 | Omitted | |
| DL-Histidine | M/8,550 | As in List I | |
| DL-Serine | M/10,000 | M/525 | Dissolved separately in distilled water and Seitz filtered. |
| DL-Threonine | M/20,000 | M/595 | |
| DL-Tryptophan | M/12,500 | Omitted | |
| DL-Methionine | M/10,000 | M/745 | |
| L-Cystine | M/30,000 | M/600 | Dissolved in HCl, Seitz filtered and neutralized after addition to the the medium. |

*Inorganic salts.*

Lists I and II.

$MgSO_4.7H_2O$ . . . Dissolved in water and autoclaved.

$FeSO_4.(NH_4)_2SO_4.6H_2O$ . Dissolved in M/115,000 citric acid and autoclaved.

*Chemical methods.*

*Reducing sugars* (and sucrose after acid hydrolysis) were estimated by the method of Hagedorn and Jensen (1923), after cultures had been cleared by precipitation with $Zn(OH)_2$.

*Lactic acid* was estimated by the method of Friedemann, Cotonio and Shaffer (1927) after cultures had been cleared by copper-lime precipitation.

*Respiration* was studied in Warburg constant volume manometers as described by Dixon (1943), using cell suspensions washed twice in M/30 phosphate buffer pH 7·6. Reaction mixtures were buffered with M/90 phosphate buffer pH 7·6 and approximately 1 mg. (dry weight) of cells shaken in 3 ml. of fluid. Oxidation quotients are expressed as $Q_{O_2} = \mu l.\ O_2/mg.$ dry wt./hr.

*Hydrogen peroxide* was detected by the method of Schales (1938) as described previously (Jordan, 1952).

## RESULTS.

*Growth in the presence of glucose as the sole carbon source.*

The initial medium tested for aerobic growth contained all the common naturally-occurring amino-acids, a wide range of growth factors, including haematin, and iron and magnesium (List I). Glucose was added at a concentration of M/80 as a source of carbon. Good growth was sometimes obtained from a small inoculum($10^3$ cells/ml.) in 18 to 30 hours, but this was not a constant finding. Often no turbidity appeared, nor did it do so if incubation was prolonged for a week. A series of growth curves showed that growth always commenced, but stopped at varying levels frequently before the culture became turbid (compare Fig. 1 and 2).

These results did not appear to be due to a nutritional deficiency. Although the frequency with which visible growth was obtained was sometimes increased, the irregularities were not entirely eliminated by increasing the concentrations of growth factors, the addition of a number of other factors or by the addition of protein-free extracts of a large number of biological materials including yeast and liver. Furthermore, filtrates from cultures in which growth had occurred, or disintegrated cells prepared from such cultures by shaking washed suspensions with glass beads (Mickle, 1948), were without effect. Growth was not limited by too low a concentration of glucose. Not more than half the added glucose disappeared from cultures even when turbid growth was obtained (Table III).

Cultivation at a low oxygen tension increased the incidence of growth. For example, in 22 parallel tests visible growth occurred 14 times when 10 ml. cultures were incubated in test-tubes, but only 3 times in cultures incubated in Erlenmeyer flasks, in which the surface area of cultures is over 10 times greater. When incubated anaerobically visible growth took place in all cultures, but only after 3 to 4 days' incubation. Not only was growth slower anaerobically, but it also never reached the density that was sometimes obtainable aerobically. A number of reducing agents—ascorbic acid, cysteine, glutathione, sodium sulphite and sodium thiolacetate—were tested but did not increase the incidence of growth. Catalase was also without effect.

Visible growth occurred more frequently from large inocula than from small ones, but anomalous results were occasionally obtained and the amount of growth was variable (Table I). These cells gave the same irregular results on subculture.

Growth on plates prepared from this medium by adding 1·5 per cent agar was as variable as in the liquid medium, the colony count varying from 0 to 90 per cent of that obtained on control TMA plates containing 50$\mu$g. haematin per ml.

TABLE I.—*The Irregularity of Growth in a Liquid Medium containing Glucose.*

| Inoculum (cells/ml.). | Growth in 36 hr. (approx. number of cells/ml. × $10^7$). Experiment. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| $10^8$ . . | 20 | 20 | 0 | 20 | 10 | 20 | 50 | 20 | 0 | 20 |
| $10^6$ . . | 20 | 20 | 0 | 10 | 5 | 20 | 20 | 0 | 0 | 20 |
| $10^4$ . . | 10 | 10 | 0 | 0 | 5 | 20 | 20 | 0 | 20 | 0 |
| $10^2$ . . | 0 | 5 | 0 | 0 | 0 | 5 | 10 | 0 | 0 | 0 |

Medium : List I with M/80 glucose.

*The presence of a toxic factor in media containing glucose.*

When glucose was omitted from solid media colony counts did not differ significantly from those on TMA-haematin plates (Tables II), although colonies

TABLE II.—*The Effect of Glucose and Lactic Acid upon Colony Counts.*

| Medium. | Number of colonies/plate. |
|---|---|
| Basal medium . . . . . | 486 |
| ,, ,, + lactic acid M/80 . . . | 544 |
| ,, ,, + glucose M/80 . . . | 237 |
| Control : TMA-haematin 50 $\mu$g./ml. . . . | 483 |

Basal medium : List I with 1·5 per cent agar. Each result is the mean of counts on 3 plates.

were much smaller in size and grew more slowly than in the presence of glucose. When glucose was replaced by M/80 lactic acid counts were not reduced below the control values and colonies were almost as large as on glucose plates.

In liquid media, growth as measured by turbidity occurred consistently from small inocula in the presence of lactic acid. No growth, as determined by viable counts, occurred in the absence of both glucose and lactic acid.

The low irregular counts obtained on addition of glucose to a solid medium suggested that it might be responsible for the presence of some toxic factor. The toxic effect was not eliminated by repeated recrystallisation of the glucose or by the use of other samples, thus indicating that the effect was dependent upon glucose itself and not upon an impurity associated with it.

*The effect of lactic and pyruvic acids on growth in a glucose medium.*

In an attempt to demonstrate the formation of a toxic factor in glucose media, a filtrate from a glucose culture in which some growth had occurred was added to medium containing lactic acid as a source of carbon. This was then inoculated. However, the filtrate increased the amount of growth in the culture containing lactic acid. As growth in a medium containing glucose always stopped before the latter was used up, the effect of adding glucose itself to lactic acid cultures was tested.

It was found that growth in a mixture of glucose and lactic acid occurred consistently and was heavier than in the presence of either alone. This effect

was not due to better pH control in the presence of lactate, because in fact no change in pH occurred during growth in a medium containing glucose. Neither was it the result of limitation of growth by too low a concentration of the carbon source present. Results in Table III show that in the basal medium used only

TABLE III.—*Growth in Liquid Media containing Glucose and Lactic Acid.*

| Glucose. | | Lactic acid. | | Growth (approximate number of cells/ml. × $10^7$). |
|---|---|---|---|---|
| Initial concentration. | Concentration utilized. | Initial concentration. | Concentration utilized. | |
| 0 | 0 | M/40 | M/95 | 20 |
| M/1250 | M/2780 | M/40 | M/83 | 20 |
| M/500 | M/1430 | M/40 | M/105 | 20 |
| M/200 | — | M/40 | — | 100 |
| M/80 | M/89 | M/40 | M/93 | 100 |
| M/80 | M/100 | M/100 | M/167 | 100 |
| M/80 | M/114 | M/250 | M/463 | 50 |
| M/80 | M/107 | M/625 | M/1150 | 10 |
| M/80 | M/210 | 0 | 0 | 5 |

Basal medium : List II. Cultures were incubated for 5 days.

about 40 per cent of the lactic acid or glucose present disappeared during growth when either was added separately. Moreover growth could be increased by the addition of relatively small quantities of lactic acid, of which only about half disappeared.

A growth curve (Fig. 1) showed a break in the rate of multiplication in a medium containing glucose and lactic acid, which resembled the phenomenon

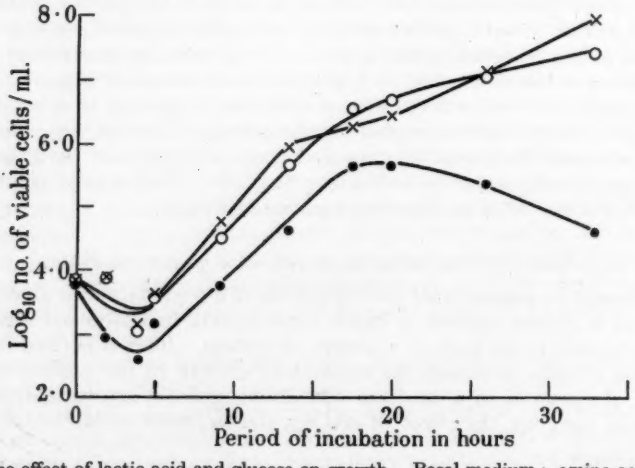


FIG. 1.—The effect of lactic acid and glucose on growth. Basal medium ; amino-acids as in List I, growth factors as in List II. Additions: × M/80 lactic acid and M/80 glucose, ○ M/80 lactic acid, ● M/80 glucose.

of " diauxie " described by Monod (1942) for the growth of some bacteria in mixtures of sugars. He concluded that such sugars were metabolised in sequence.

Results on solid media confirmed those obtained in liquid media, the addition of M/40 lactic acid to plates containing M/80 glucose preventing the low counts usually obtained on such plates. Colonies were larger than in the presence of either separately. This result was obtained even when the combined concentration of glucose and lactic acid was not greater than that of either alone.

Pyruvic acid had an effect on growth similar to that of lactic acid. It permitted regular and heavy growth in liquid media containing glucose, and prevented the reduction in colony counts on plates containing glucose.

*The effect of other sugars on growth.*

Acid was formed by this strain of *Past. septica* when it was grown in 2 per cent Eupeptone containing 1 per cent of sucrose, glucose, fructose and galactose. The effects of these sugars on growth were therefore compared. Maltose was also tested, for although it was not fermented in peptone water, it was oxidised at a rate comparable with the oxidation of glucose (Table VII).

Table VI shows that galactose and maltose had the same effect on aerobic colony counts as glucose, only a proportion of the cells seeded developing into colonies when these sugars were included in the medium.

On the other hand, when sucrose or fructose were added to the media as sources of carbon, colony counts did not differ significantly from the control values (Table IV). Colonies on plates containing sucrose were fairly large, and

TABLE IV.—*The Effect of Sucrose and Fructose on Colony Counts.*

| Additions to the medium. | | | Number of colonies/plate. |
|---|---|---|---|
| Sucrose (M/80). | Fructose. (M/80). | Glucose. (M/80). | |
| + | 0 | 0 | 1782 |
| 0 | + | 0 | 1737 |
| 0 | 0 | + | 1062 |
| + | 0 | + | 1587 |
| 0 | + | + | 1943 |
| Control : TMA-haematin | | | 1790 |

Basal medium : List I with 1·5 per cent agar. Each result was the mean of counts on 2 plates.

visible growth in the comparable liquid medium occurred regularly although it was relatively slow. When fructose was added to plates, colonies were no larger than in its absence. Little or no growth occurred in the equivalent liquid medium, as can be seen from Fig. 2, the result being the same as that obtained when no energy source was added, other than amino-acids. However, Table IV shows that fructose as well as sucrose, when added to a medium containing glucose, would prevent the reduction in colony counts caused by the latter. Growth in a liquid medium containing glucose was increased when sucrose or fructose was also included, the effect of the latter being shown in Fig. 2. The effect of sucrose on growth in a medium containing glucose was presumably due to its fructose content, as during the growth of a culture it was converted to reducing sugar, which later disappeared (Table V).

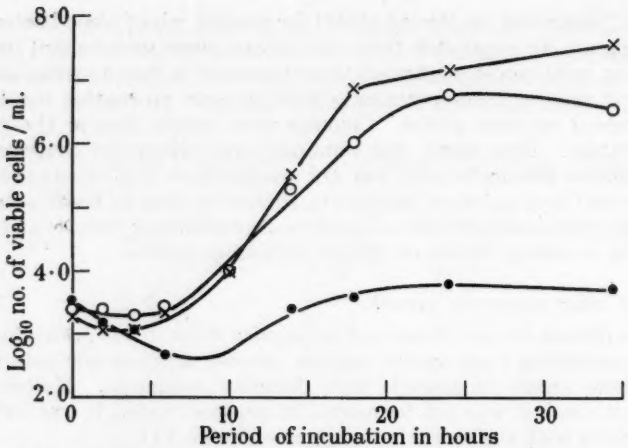


FIG. 2.—The effect of glucose and fructose on growth. Basal medium: amino-acids as in List I, growth factors as in List II. Additions: × M/80 glucose and M/80 fructose, ○ M/80 glucose, ● M/80 fructose.

TABLE V.—*The Catabolism of Sucrose during Growth.*

| Incubation (days). | Growth (approx. number of cells/ml. × $10^7$). | Decrease in sucrose concentration (per cent). | Sucrose present as reducing sugar (per cent). |
|---|---|---|---|
| 2 | 10 | 58 | 59 |
| 3 | 50 | 63 | 38 |
| 6 | 50 | 92 | 18 |

Basal medium: List II with sucrose M/80.

It was not determined whether glucose or glucose-1-phosphate was the intermediary formed during sucrose metabolism. No information could be obtained from the use of glucose-1-phosphate, as when this was added to a liquid medium as a source of carbon the lag phase was increased from about 8 to 16 hours. It is possible that its hydrolysis was required before its absorption.

*Anaerobic growth.*

It has already been mentioned that when cultures were incubated anaerobically growth occurred consistently in glucose media but was slow and stopped at a low level. When plates were incubated anaerobically, it was found that counts on media containing glucose or galactose were not significantly lower than the control values (Table VI).

TABLE VI.—*The Effect of Galactose, Maltose and Glucose on Colony Counts.*

| Additions to the medium (M/80). | Mean count on 4 plates. Aerobic. | Anaerobic. |
|---|---|---|
| Galactose | 50 | 402 |
| Maltose | 46 | — |
| Glucose | 56 | 442 |
| Control: TMA-haematin | 416 | |

Basal medium: List II with 1·5 per cent agar.

*The production of hydrogen peroxide during the oxidation of sugars.*

As glucose and galactose did not inhibit anaerobic growth, it was considered possible that hydrogen peroxide was formed aerobically during the oxidation of these sugars in concentrations sufficient to inhibit growth, in spite of the presence of haematin and bacterial catalase. Furthermore, as maltose was not fermented, it was presumably a product of oxidative metabolism which caused inhibition by this sugar. The low variable counts on glucose plates and the failure of attempts to train organisms to grow regularly on glucose media resembled the results obtained on TMA lacking haematin, which were attributed to inhibition by hydrogen peroxide (Jordan, 1952).

The production of hydrogen peroxide during growth could not be demonstrated either by testing samples of cultures with the phenolphthalein reagent of Schales (1938) or by the inclusion of manganese dioxide in the medium (Kneteman, 1947). However, as all the sugars under consideration and also lactic acid were oxidised by resting cell suspensions, it was possible to test for the production of hydrogen peroxide under conditions in which its formation would be rapid and catalase production minimal. The respiratory activity of cell suspensions was very variable, but the relative rates of oxygen uptake with different substrates were constant. Between 1 and 10 $\mu$g. hydrogen peroxide/ml. was detectable by the oxidation of phenolphthalein at the end of one hour's shaking with glucose, maltose or galactose. Traces of peroxide were also formed during the oxidation of sucrose and fructose, but none could be detected as a result of lactic acid oxidation (Table VII). When pyruvic acid was added to the reaction

TABLE VII.—*The Production of Hydrogen Peroxide by Cell Suspensions.*

| Respiratory substrate (M/24). | $Q_{O_2}$. | Hydrogen peroxide formed ($\mu$g./ml.). |
|---|---|---|
| O | 18 | $<0{\cdot}01$ |
| Lactic acid | 184 | $<0{\cdot}01$ |
| Glucose | 174 | Approx. 10 |
| Fructose | 114 | ,, 0·1 |
| Sucrose | 54 | ,, 0·1 |
| Galactose | 90 | ,, 10 |
| Maltose | 168 | ,, 10 |

mixture, no hydrogen peroxide could be demonstrated during the oxidation of other compounds. This result was not obtained with lactic acid. It therefore seemed probable that although pyruvic acid might support growth on a medium containing glucose by reacting with hydrogen peroxide as it was formed, it was unlikely that lactic acid protected the organism by acting as a source of pyruvic acid.

The oxidation of glucose was not affected by M/100 cyanide or M/100 hydroxylamine, which reduced the rate of oxidation of succinic acid by about 80 per cent (Table VIII). The insensitivity of glucose oxidation to these inhibitors indicated that under these conditions its oxidation did not involve the cytochrome system and was therefore probably catalysed by flavoprotein enzymes. Such enzymes are known to catalyse peroxide-producing reactions. Cyanide and hydroxylamine both inhibit catalase. The former interferes with the reagent for detecting

hydrogen peroxide, but after oxidation of glucose in the presence of the latter the amount of hydrogen peroxide present was found to be increased (Table VIII).

TABLE VIII.—*The Effect of Inhibitors on the Oxidation of Glucose and Succinic Acid.*

| Substrate. | | Inhibitor. | | | |
|---|---|---|---|---|---|
| Glucose (M/30). | Succinic acid (M/30). | Cyanide (M/100). | Hydroxylamine (M/100). | $Q_{O_2}$. | Hydrogen peroxide formed (μg./ml.). |
| + | — | — | — | 28 | Approx. 1 |
| + | — | + | — | 34 | — |
| + | — | — | + | 25 | Approx. 5 |
| — | + | — | — | 194 | <0·01 |
| — | + | + | — | 40 | — |
| — | + | — | + | 25 | <0·01 |

Succinic acid (E. Merck) was neutralised before use.

## DISCUSSION.

The depression of bacterial growth on inclusion of glucose in the culture medium has been described for a few organisms, but where the mechanism has been investigated it has been associated with a reduction in protein metabolism (Kligler and Grossowicz, 1942; Shaposhnikov and Tauson, 1948). Although no report has been found of inhibition of growth by hydrogen peroxide produced as a by-product of glucose oxidation, lactobacilli have been described which catalyse the formation of hydrogen peroxide in this matter (Bertho and Glück, 1932; Davis, 1933). The results recorded here suggest that the oxidations of glucose, galactose and maltose are of this type, and that hydrogen peroxide may accumulate during their metabolism in quantities sufficient to inhibit growth in spite of the presence of catalase and haematin.

Further investigations are required to explain the ability of sucrose, fructose and lactic acid to counteract this inhibition. However, the partial nature and the variability of the inhibition by glucose suggested that the amount of growth occurring in such conditions depended upon the relative rates of hydrogen peroxide and catalase formation during growth. It is possible that the provision of an alternative source of carbon might permit growth and catalase formation to occur sufficiently rapidly relative to glucose oxidation to prevent inhibition.

## SUMMARY.

In a chemically-defined medium which will support the growth of a strain of *Past. septica*, lactic acid or sucrose are adequate as sources of carbon. Glucose, maltose and galactose inhibit aerobic growth to a variable extent when added as the only carbon sources. Evidence is presented that this inhibition is caused by the production of toxic quantities of hydrogen peroxide during the oxidation of these sugars by the organism. No inhibition is obtained if glucose is added to a medium with another source of carbon, and in fact heaviest growth is obtained in a medium containing glucose and lactic acid in equal proportions.

My thanks are due to Dr. G. P. Gladstone, who supervised this work, for much helpful advice and criticism. I also wish to thank the Medical Research Council for a Studentship.

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# INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI. PRELIMINARY EXAMINATION OF THE ELEVENTH 100 SPECIES, ALL BASIDIOMYCETES.

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THE continuation of the above work is the eleventh paper in the series and the seventh of the papers which have dealt only with Basidiomycetes. The cultures were isolated by ourselves, as indicated by " W" in front of the number, grown on the two standard media, potato dextrose and malt, and the metabolism solutions were tested for anti-bacterial activity by the " Wilkins Revised " method (Wilkins. 1949) against the representative types *Bacterium coli* and *Staphylococcus aureus*. The results are arranged in the usual three groups, " strongly positive," " weakly positive " and " negative "; only the strongly positive are listed in detail.

## LIST OF FUNGI EXAMINED.

### *Strongly Positive.*

| | Result of test on liquid medium. | | | |
|---|---|---|---|---|
| | Time in days. | Medium.* | *Bact. coli.* | *Staph. aureus.* |
| *Clitocybe aurantiaca* (Wulf.) Studer W682A | 35 | M | — | 20 |
| *C. aurantiaca* (Wulf.) Studer W682B | 15 | PD | 18 | 28 |
| *Collybia butyracea* (Bull.) Fr. W610D | 10 | PD | 21 | 31 |
| *Coprinus picaceus* (Bull.) Fr. W616C | 15 | M | — | 22 |
| *Fomes annosus* Fr. W698B | 15 | PD | 21 | 29 |
| *Inocybe geophylla* (Sow.) Fr. W675A | 10 | PD | — | 23 |
| *Poria xantha* Lind. W6104A | 15 | M | 19 | 20 |
| *P. xantha* Lind. W6104B | 25 | PD | 18 | 20 |
| *Psalliota xanthoderma* Genev. W677B | 35 | M | 25 | 24 |
| *P. xanthoderma* Genev. W677G | 20 | M | 19 | 20 |

* M = malt; PD = potato dextrose.

### *Weakly Positive.*

*Clavaria cinera* (Bull.) Fr. W672A; *Clitocybe aurantiaca* (Wulf.) Studer W682C; *C. diatreta* Fr. W674A; *C. inversa* (Scop.) Fr. W691A; *Cyathus striatus* (Huds.) Pers. W639A; *Leptonia incana* Fr. W6170A; *Marasmius oreades* (Bolt.) Fr. W655D; *Pleurotus sapidus* Schulz. W664B; *Psalliota xanthoderma* Genev. W677D; *Polyporus betulinus* (Bull.) Fr. W620D; *P. lacteus* Fr. W6142A; *P. schweinitzii* Fr. W6145A; *P. sulphureus* (Bull.) Fr. W634C; *Scleroderma aurantium* Pers. W637A.

### *Negative.*

*Armillaria mellea* (Vahl.) Fr. W688C; *Boletus crocipodius* Letell. W6151A; *B. granulatus* (Linn.) Fr. W6118A; *Clitocybe aurantiaca* (Wulf.) Stuber W682F; *C. ditopus* Fr. W650A; *C. infundibuliformis* (Schaeff.) Fr. W668A; *C. inversa* (Scop.) Fr. W691B and W691C; *Collybia fusipes* (Bull.) Berk. W662D; *C. platyphylla* (Pers.) Fr. W6147A; *Coprinus atramentarius* (Bull.) Fr. W640B;

*C. micaceus* (Bull.) Fr. W6110B and W6110C ; *C. niveus* (Pers.) Fr. W653A ; *C. plicatilis* (Curt.) Fr. W659A ; *Cortinarius sanguineus* (Wulf.) Fr. W63A ; *Daedalea biennis* (Bull.) Quél. W6132A ; *D. quercina* (Linn.) Fr. W649B ; *Fistulina hepatica* (Huds.) Fr. W632B ; *Flammula sapinea* Fr. W624A ; *Fomes fraxineus* (Bull.) Fr. W6126A ; *F. igniarius* (Linn.) Fr. W6125A ; *Ganoderma applanatum* (Pers.) Pat. W6103B ; *G. resinaceum* Boud. W6143A ; *Hypholoma candolleanum* Fr. W6148A ; *H. fasciculare* (Huds.) Fr. W635C and W635D ; *H. lacrymabundum* Fr. W6128A ; *Lepiota cristata* (A. & S.) Fr. W6165A ; *L. procera* (Scop.) Fr. W625B ; *Lycoperdon pyriforme* (Schaeff.) Pers. W6157A ; *Marasmius dryophilus* (Bull.) Karst W636A and W636B ; *M. erythropus* (Pers.) Fr. W687B ; *M. oreades* (Bolt.) Fr. W655B and W655C ; *M. peronatus* (Bolt.) Fr. W613C ; *Merulius tremellosus* (Schrad.) Fr. W6117A ; *Mycena epipterygia* (Scop.) Fr. W612B and W612D ; *M. vitilis* Fr. W633A ; *Pholiota spectabilis* Fr. W6144A ; *P. squarrosa* (Mull.) Fr. W6169A ; *Pleurotus sapidus* Schulz. W664D ; *Pluteus cervinus* (Schaeff.) Fr. W660B, W660C and W660D ; *Polyporus adustus* (Wild.) Fr. W629B ; *P. betulinus* (Bull.) Fr. W620E and W620F ; *P. hispidus* (Bull.) Fr. W6123A ; *P. intybaceus* Fr. W6122A ; *P. stipticus* (Pers.) Fr. W6129A ; *P. sulphureus* (Bull.) Fr. W634B ; *Polystictus versicolor* (Linn.) Fr. W627B and W627E ; *Psalliota arvenis* (Schaeff.) Fr. W617W ; *P. augusta* Fr. W622B ; *P. sylvatica* (Schaeff.) Fr. W669C ; *P. sylvicola* (Vitt.) Fr. W667B, W667C, W667F, W667G and W667H ; *P. villatica* (Brond.) Magn. W673A and W673D ; *P. xanthoderma* Genev. W677A, W677F, W677H, W677K and W677L ; *P. xanthoderma* Genev. var. *grisea* Pearson W679A and W679B ; *P. xanthoderma* Genev. var. *obscurata* Maire W678A ; *Tricholoma personatum* Fr. W62A ; *T. saponaceum* Fr. W661A.

It was stated in the previous paper which dealt with the sixth 100 Basidiomycetes (Wilkins and Partridge, 1950) that, generally speaking, of the total number of fungi examined 50 per cent showed a positive (SP and WP taken together) and 50 per cent showed a negative reaction. The above results fall below the usual standard in that the total number of " positives " is only 24 per cent. Recent results have tended towards the opinion that 50 per cent positives must be taken as the upper limit of expectation rather than as an average.

I regret to have to make a correction. *Lenzites thermophila* Falck 75 in the SP list (Wilkins, 1947a) and *L. thermophila* Falck 75A in the WP list (Wilkins, 1947b) are not merely the same species but, though received from different sources, prove to be duplicate isolations from the same fungus. Moreover, the correct name of the fungus is *Lenzites trabea* (Pers.) Fr. 108 (Cartwright 1931). From a culture of the first named, i.e., 75, Burton (1950) of the Oxford School of Pathology extracted an antibiotic which he called "Thermophillin." I have apologised to Dr. Burton (Wilkins, 1950) for misleading him as to the name when I supplied the culture, but wish to repeat the correction.

My best thanks are due to the Medical Research Council for continuation of the grant enabling me to employ Research Assistants, and I am grateful for valuable help from Miss Elizabeth Turner, M.A., Ph.D., Miss June Rees, B.Sc., Miss Jill Rose and Mrs. S. D. Edwards.

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# SEROLOGICAL STUDIES ON THE ANTIGENICITY AND PURITY OF ADRENOCORTICOTROPHIC HORMONE AND GROWTH HORMONE.*

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SINCE the isolation of growth hormone and adrenocorticotrophic hormone (ACTH) as physically and chemically homogeneous substances, the metabolic effects of these hormones have been extensively studied. Little attention, however, has been paid to their possible antigenic properties. Chase (1949) injected mice over a period of 25 days with ACTH, and found that sera from such mice possessed both agglutinins and precipitins. The injection of these sera into normal mice prevented the normal action of ACTH in increasing the adrenal gland weight. Gordon (1949) showed that rats receiving repeated injections of ACTH gradually became refractory to a test dose of the hormone which depleted the adrenal ascorbic acid in the control animals. This worker also found that although antibodies could not be detected in the sera, the anti-ACTH factor could be passively transferred to mice. In connection with these two results, it is worthy of note that the ACTH used by Chase was prepared by the method of Sayers, White and Long (1943), and that the preparation was homogeneous, as judged by its behaviour in the Tiselius electrophoresis apparatus and in the ultracentrifuge. Gordon (1949), on the other hand, used an Armour preparation, which had only 35 per cent of the biological activity of the La-1-A standard ; no data were given as to the homogeneity of the preparation. Anderson, Page and Li (1947) showed that the hypertension of hypophysectomized rats treated with an electrophoretically homogeneous preparation of ACTH could not be maintained after ten days in spite of the continued injection of the hormone. This result could be explained as being due to a true formation of antibodies or to an increased tolerance to the hormone.

Elberg and Li (1950) found that repeated injections of crystalline growth hormone did not induce the appearance of precipitating antibodies in the rabbit or anaphylactic sensitization in the guinea-pig. Simpson and Li (1948) had previously shown that the administration of growth hormone for 438 days resulted in continuous growth in hypophysectomized rats and overgrowth in normal rats. This suggested that neutralizing antibodies if present exerted no protective influence in animals receiving the hormone for long periods. The only report, so far as we are aware, to suggest that growth hormone is antigenic comes from Leathem (1949). Leathem found that serum taken from a patient who, at first, responded to growth hormone, but later became refractory, was able to inhibit the growth rate of hypophysectomized rats treated with growth hormone.

* A preliminary report of this work was given to the Biochemical Section of the A.N.Z.A.A.S. meeting at Brisbane, Australia, in May, 1951.

As part of a programme designed to determine whether or not the entire fat-mobilizing activity of the anterior pituitary gland is due to its content of ACTH and growth hormone, we have reinvestigated the antigenicity of the two hormones. The initial results led us to suspect the formation of antibodies to growth hormone but not to ACTH, and have been reported (Morrison, Ennor, Bazeley and Thayer, 1952). Since this work was submitted for publication further work on the problem has been done, and it is the purpose of this paper to detail the findings which indicate that growth hormone, but not ACTH, is antigenic.

## MATERIALS AND METHODS.

ACTH was prepared by the method of Fishman (1947) from freshly collected and frozen hog pituitary glands. Growth hormone was prepared in a crystalline form from the anterior lobes of freshly collected and frozen ox pituitary glands, according to Wilhelmi, Fishman and Russell (1948). To differentiate this preparation from another given to us by Dr. Wilhelmi, it will be referred to as Australian National University (A.N.U.) growth hormone. Both our hormone preparations were physiologically active, and behaved as single components in the Tiselius electrophoresis apparatus. The protein N contents of the ACTH and of the growth hormone were 15·5 and 15·7 per cent respectively.

Crude extracts of whole hog (hog P.E.) and sheep pituitary glands (sheep P.E.) and of the anterior lobes of ox pituitaries (ox A.P.E.) were obtained by grinding the tissue with coarse silica in the presence of 0·9 per cent NaCl and stirring at 0° for one hour. The extracts were quickly centrifuged to remove easily sedimented material and then centrifuged for one hour at 34,000 **g** at 0°. The supernatant fluid was removed with aseptic precautions, aliquots taken for the determination of protein N and the final volume adjusted with 0·9 per cent NaCl to bring the protein N content to 2·0 mg./ml. The extracts were stored at 0° in the presence of 0·1 per cent phenol. Under these conditions, the solutions did not lose hormonal activity over a period of some weeks. The pure hormones were made up in 0·9 per cent NaCl and brought to pH 8·0 prior to injection.

For the preparation of immune sera English hutch rabbits of body weight *ca.* 1·5 kg. were injected intraperitoneally with gradually increasing doses (1–4 mg.) of pure ACTH and growth hormone at 5-day intervals. With crude extracts each dose (4 ml.) was equivalent to 8 mg. of protein N. For the small volume of sera required for preliminary titrations blood was taken from the marginal ear vein ; larger volumes were collected by cardiac puncture. The serum, after separation, was centrifuged and stored at 0° in the presence of 0·1 per cent phenol.

For precipitin tests all extracts and pure hormone preparations were diluted with 0·9 per cent NaCl and brought to pH 7·2. Unless otherwise stated, dilutions of sera were made in a 4 per cent aqueous solution of horse albumin, which was prepared according to the method described by Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor (1946). It has been found (Bazeley and Thayer, unpublished observations) that dilutions made in this manner lead to greater sensitivity and reproducibility of the precipitin reaction. In the experiments requiring absorption of the serum, the requisite volume of the antigen was freeze-dried and the resultant material mixed with an appropriate aliquot of the undiluted serum. The mixture was allowed to stand for 3 hours at 37° and then clarified by high-speed centrifugation at 0°. Greater sensitivity in the precipitin test was achieved by placing the test solution over the serum layer with a fine

capillary pipette so that the fluid interface was formed with minimal mixing. The tests were carried out at room temperature (20°–25°) and the interfacial reaction read after 2 hours.

## RESULTS.

### *ACTH.*

Titrations carried out on sera obtained from rabbits given repeated injections of ACTH over 9 weeks failed to demonstrate specific precipitins, even though ox A.P.E., hog P.E. and ACTH were used as test antigens.

In these experiments two samples of ACTH were used—one prepared by us, the other given to us by Messrs. Armour & Company, of Chicago. Since negative results were obtained with the four test antigens, it is clear that under the conditions of our experiment ACTH is non-antigenic. The negative result obtained when hog P.E. was the test antigen is of interest, as it indicates freedom from contamination of the ACTH by any antigenic hog protein.

The pooled sera from another group of rabbits which had received 8 injections of hog P.E. were titrated against the same test antigens. The results (Table I)

TABLE I.—*Precipitin Titres of Serum from Rabbits Injected with ACTH and Hog P.E.*

Rabbits injected with ACTH received 42 mg. over a period of 9 weeks; rabbits injected with Hog P.E. received 5 injections equivalent to 40 mg. protein nitrogen.

| Test antigen. | ACTH Undiluted serum. | Hog P.E. Dilution of rabbit serum. | | | | |
|---|---|---|---|---|---|---|
| | | Undiluted. | 1/2. | 1/4. | 1/8. | 1/16. |
| ACTH (1 mg./ml.) | — | — | — | — | — | — |
| Armour ACTH (1 mg./ml.) | — | — | — | — | — | — |
| Hog P.E. | — | +++ | ++ | ++ | + | — |
| Ox A.P.E. | — | ++ | + | — | — | — |

confirm the above conclusions and, in addition, suggest that in the hog pituitary gland there are present antigens which are also common to the anterior lobe of the ox pituitary. Some indication of this was obtained by Ennor and Singer (unpublished observations), and will be referred to below in the experiments dealing with growth hormone.

### *Growth hormone.*

The preliminary titrations (Table II) of sera obtained from rabbits injected with varying amounts of growth hormone are in accord with what might be

TABLE II.—*Precipitin Titres of Serum from Rabbit Injected with Growth Hormone.*

| Test antigen. | Number of injections. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5. | 6. | 7. | 8. | 9. | 10. | 12. | 13. |
| A.N.U. growth hormone | ++++ | ++++ | ++ | ++++ | +++ | +++ | ++++ | +++ |
| Hog P.E. | ++ | + | — | ++ | — | + | — | .. |
| Sheep P.E. | .. | .. | .. | .. | ++ | .. | ++ | .. |
| Ox A.P.E. | ++++ | ++++ | ++ | ++++ | ++++ | +++ | ++ | .. |

expected if this hormone was, in fact, antigenic. When tested against both the homologous antigen and ox A.P.E. strongly positive precipitin reactions were obtained as early as after the fifth injection. Positive results, although less intense, were obtained with sheep and hog P.E. as test antigens, confirming previously observed non-specific reactions due to anterior lobe extract. The rise and fall in intensity of the observed reactions is common to all the test antigens used.

While these results indicated the antigenicity of growth hormone, the strongly positive reactions obtained when ox A.P.E. was the test antigen suggested the contamination of the hormone with other antigenic proteins of ox tissue. This possibility received some support from the results (Table III) which showed

TABLE III.—*Precipitin Titres of Serum from Rabbits Injected with Growth Hormone.*

Rabbits received 10 injections equivalent to 30 mg. hormone over a period of 7 weeks.

| Test antigen. | Dilution of rabbit serum in normal rabbit serum. | | | |
|---|---|---|---|---|
| | N. | 1/2. | 1/4. | 1/8. |
| Growth hormone . . . | +++ | +++ | + | — |
| Hog P.E. . . . . | + | — | — | — |
| Ox A.P.E. . . . . | +++ | ++ | + | — |

identical titres for both growth hormone and beef A.P.E. To elucidate this point, antigens prepared from the adrenal gland, skeletal muscle and brain of the ox, sheep and hog were tested against the antisera. In the case of each species equal weights of the tissues were taken, the extract prepared and the protein N content adjusted, as before, to give an N content similar to that chosen in the case of the crude pituitary extracts. In addition, we were fortunate in securing a second sample of growth hormone to use as a test antigen—one which was kindly supplied by Dr. Wilhelmi—Wilhelmi Growth Hormone.

The results of the precipitin tests carried out on sera from three series of animals injected with hog P.E., growth hormone and ox A.P.E. are shown in Table IV. Though there was a definite anti-hog titre in the sera of the animals injected with hog P.E., there was no evidence of antibodies to either of the growth hormone preparations. Sera from animals injected with growth hormone and with ox A.P.E. gave positive reactions to the injected antigens and also to the ox tissue extract and in a lesser degree to the sheep and hog tissue extracts. These results suggested that our growth hormone preparation (and Dr. Wilhelmi's) was antigenic, but in view of the positive reactions with ox tissue extract, there remained the possibility that the preparation contained some non-specific antigenic protein contaminant.

Table V illustrates the results of an experiment designed to check this possibility. The serum obtained from a rabbit injected with growth hormone over a period of nine weeks (total dose = 42 mg.) was titrated against all the test antigens, both before and after absorption with ox tissue extract. Such absorption removed all the antibodies which interact with ox tissue proteins, but did not reduce the intensity of the reaction to either of the two growth hormone preparations used as test antigens. It would thus seem certain that growth hormone is antigenic and, in addition, since positive reactions have been obtained

TABLE IV.—*Precipitin Titres of Serum from Rabbits Injected with Hog P.E., Growth Hormone and Ox A.P.E.*

| Test antigen. | Hog P.E. (five injections). Dilution of serum. | | | | | | Growth hormone (nine injections). Dilution of serum. | | | | Beef A.P.E. (eight injections). Dilution of serum. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N. | 1/2. | 1/4. | 1/8. | 1/16. | 1/32. | N. | 1/2. | 1/4. | 1/8. | N. | 1/2. | 1/4. | 1/8. |
| Wilhelmi growth hormone | − | − | − | − | − | − | ++ | − | − | − | ++ | + | + | − |
| A.N.U. growth hormone | − | − | − | − | − | − | +++ | ++ | + | − | ++ | − | − | − |
| Beef A.P.E. | + | + | − | − | − | − | ++ | + | − | − | ++++ | ++ | + | − |
| Hog P.E. | ++++ | +++ | ++ | + | + | − | − | − | − | − | − | − | − | − |
| Sheep P.E. | + | − | − | − | − | − | ++ | + | − | − | +++ | ++ | + | − |
| Hog tissue extract | +++ | ++ | + | − | − | − | − | − | − | − | − | − | − | − |
| Ox tissue extract | − | − | − | − | − | − | ++ | ++ | + | − | ++ | − | − | − |
| Sheep tissue extract | + | − | − | − | − | − | + | − | − | − | + | − | − | − |

TABLE V.—*Titration of Growth Hormone Antiserum* before and after Absorption with Ox Tissue Extract.*

1·5 ml. serum was absorbed with the solids from 0·1 ml. beef tissue extract.
Absorption period was 3 hours at 37°.

| Test antigen. | Before absorption. Serum dilution. | | | | After absorption. Serum dilution. | | | |
|---|---|---|---|---|---|---|---|---|
| | N. | 1/2. | 1/4. | 1/8. | N. | 1/2. | 1/4. | 1/8. |
| Ox tissue extract | ++ | ++ | + | − | − | − | − | − |
| Ox A.P.E. | ++ | + | − | − | + | − | − | − |
| A.N.U. growth hormone | ++ | ++ | + | − | ++ | + | + | − |
| Wilhelmi growth hormone | ++ | − | − | − | ++ | + | − | − |
| Hog P.E. | .. | .. | .. | .. | + | − | − | − |
| Hog tissue extract | .. | .. | .. | .. | − | − | − | − |
| Sheep P.E. | .. | .. | .. | .. | ++ | + | + | − |
| Sheep tissue extract | .. | .. | .. | .. | − | − | − | − |

* Serum was identical with that referred to in Table IV.

TABLE VI.—*Titration of Growth Hormone Antiserum after Absorption with Growth Hormone.*

The serum was absorbed with beef tissue extract prior to absorption with growth hormone preparations.

| Test antigen. | 1 ml. serum absorbed with A.N.U. growth hormone. | | | | | 1 ml. serum absorbed with Wilhelmi growth hormone. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Absorbed with 320 μg. | Absorbed with 80 μg. | | | | Absorbed with 320 μg. | Absorbed with 80 μg. | | | |
| | Serum dilution. | Serum dilution. | | | | Serum dilution. | Serum dilution. | | | |
| | undiluted. | Undiluted. | 1/2. | 1/4. | 1/8. | undiluted. | Undiluted. | 1/2. | 1/4. | |
| Ox tissue extract | − | − | − | − | − | − | − | − | − | |
| Ox A.P.E. | − | + | − | − | − | − | + | − | − | |
| A.N.U. growth hormone | − | ++ | + | + | − | − | ++ | + | − | |
| Wilhelmi growth hormone | − | ++ | + | − | − | − | ++ | (+)* | − | |

* Trace of precipitin ring only.

TABLE VII.—*Titration of Wilhelmi and A.N.U. Growth Hormone against Growth Hormone Antiserum.*

The serum was initially absorbed with beef tissue extract.

| Serum. | Quantity of growth hormone in 1 ml. of 0·85 per cent NaCl. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 500 μg. | 250 μg. | 125 μg. | 62 μg. | 31 μg. | 15 μg. | 8 μg. |
| A.N.U. growth hormone | +++ | +++ | ++ | ++ | + | + | − |
| Wilhelmi growth hormone | .. | +++ | +++ | ++ | + | + | − |

when hog P.E. and sheep P.E. were used as test antigens against this absorbed serum, that the hormone is not species-specific. It is clear from this evidence that our own preparation of growth hormone was not completely free from a trace of ox tissue contaminating antigens. The antigenicity of growth hormone is further indicated by the results of cross-absorption tests carried out with the pure hormone preparations (Table VI). The results show that whilst complete absorption was not effected with 80 μg. of growth hormone (irrespective of the preparation used), 320 μg. of the hormone was able to remove all antibodies in both cases. The absorption was also identical, irrespective of which of the two pure growth hormones was used. Both hormone preparations were titrated against an anti-growth hormone immune serum (Table VII) from which non-specific antibodies had been removed by absorption with ox tissue extract. That the two preparations are identical is suggested by the fact that the same titres were obtained.

## DISCUSSION.

Under the conditions of our experiments ACTH does not readily elicit an antibody response in the rabbit. These results are contrary to those of Gordon (1949) and Chase (1949), but these workers used rats and mice respectively, and it is possible that there is a marked species difference in sensitivity. As far as our findings are concerned, it should be remembered that ACTH has a half-life of 5·5 minutes in the blood stream (Greenspan, Li and Evans, 1950), and it is thus conceivable that the hormone did not remain intact for a sufficient length of time to stimulate the production of antibodies. Greater weight could be placed upon our results with ACTH if injections had been made at closer time intervals with or without the use of an adjuvant. Such experiments are not contemplated. It is, however, of interest to note that our preparation of ACTH is not contaminated by any antigenic protein.

The position with growth hormone is quite different. The indication is that it is antigenic and, moreover, non-species specific. It is significant that precipitating antibodies were detected not only with our preparation, but also with that supplied by Dr. Wilhelmi, particularly as the latter was prepared by a different method. It would seem unlikely, then, that these two hormone preparations were contaminated to the same degree by the same protein(s). Thus it is indicated that the antibodies were indeed produced as a result of the response of the animal to growth hormone as at present isolated. It should be borne in mind, however, that our most purified preparation still contained antigenic contaminating proteins which reacted in the precipitin test with beef tissue extract (*vide* Table IV). Unfortunately, the opportunity did not present itself to test Dr. Wilhelmi's preparation, since this would have involved immunizing the animals with this preparation, of which our supply was limited. If, however, it be accepted that growth hormone is indeed antigenic, the results quoted in Table VII are of added interest. They suggest the possibility of using a serological technique for the standardisation of the hormone, and so avoiding the rather laborious biological assay methods. Experiments designed to test this possibility are in progress.

It is perhaps surprising that growth hormone is antigenic, since such a property would seem inimical to the function of the hormone in the young animal. There is, however, no guarantee that the hormone, as isolated, is identical with that released from the anterior pituitary gland under *in vivo* conditions. It is possible

that a physiologically active moiety is attached to a carrier protein, as appears to be the case with ACTH, and it may be that the hormone exists in the pituitary gland in this combined form. In the intact animal the active moiety only is released, perhaps after hydrolysis by a proteolytic enzyme within the pituitary. On the other hand, the injection of growth hormone, as isolated, may be visualized as inducing the production of antibodies, not to the physiologically active moiety, but to the physiologically inactive carrier protein.

SUMMARY.

The antigenic properties of ACTH and growth hormone have been studied. No antibodies could be detected in the sera of rabbits which received up to 40 mg. of ACTH. This result is taken to indicate that ACTH is non-antigenic and, moreover, that the preparation used was free from contamination by antigenic hog proteins. In the case of growth hormone antibodies were detected, and it is concluded that growth hormone, as prepared in a crystalline and electrophoretically homogeneous state, is antigenic and non-species specific. Two samples of growth hormone prepared in a crystalline form by different methods and each behaving as a single component in the Tiselius electrophoresis apparatus were shown to contain traces of contaminating protein(s).

It is a pleasure to express our thanks to Messrs. Armour & Company of Chicago for a gift of ACTH, and to Dr. Wilhelmi of Emory University, Georgia, U.S.A., for a gift of growth hormone and details of a new method of preparation. Our thanks are also due to Dr. J. A. Broben of the Commonwealth Serum Laboratories for supplies of beef anterior pituitary glands, to Mr. F. Dempster of the Commonwealth Serum Laboratories for carrying out electrophoretic analyses on all the hormone preparations used in this work, and to Mr. R. Frater for skilled technical assistance. Two of us (A. H. E. and J. F. M.) are also indebted to the Director-General of Health and to the Director of the Commonwealth Serum Laboratories for a generous provision of laboratory space pending the establishment of the Department in Canberra.

Since this paper was submitted for publication our attention has been drawn to the findings of Marshall (1951), which indicate, contrary to the above findings, that ACTH prepared from hog pituitary gland is antigenic. At the moment no explanation can be put forward for the difference in experimental findings. Two points are of especial interest: (*a*) Our attempts at the production of antibodies to ACTH have been confined to the use of the intact rabbit, whereas Marshall (1951) has employed the adrenalectomized rabbit in the belief that the presence of the adrenals interferes with the immune response. Our failure to demonstrate antigenicity may well have been due to this cause. (*b*) Marshall's preparation of ACTH is clearly a mixture of proteins, and the fact that an antibody reaction was obtained only in the case of hog pituitary (the source of the ACTH), together with the demonstration of other contaminating proteins, suggests that the antibody reaction obtained was species-specific, i.e., some hog protein, other than ACTH, was involved.

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# STUDIES ON THE REPRODUCTION OF BACTERIAL VIRUSES. II. EFFECT OF ENZYME-INHIBITORS ON THE MULTIPLICATION OF THE COLIPHAGE T2.

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ATTEMPTS have been made to demonstrate enzymic activity in purified suspensions of viruses (see review by Bauer, 1949), but with little success. It is generally conceded, however, that the multiplication of the infective agents belonging to the virus class is intimately linked with the metabolism of the host-cell (Foster, Jones, Henle and Dorfman, 1944; Friedewald, 1942; Parker and Thompson, 1942; Rasmussen, Waisman, Elvehjem and Clark, 1944; Sprunt, 1942; Toomey, Frohring and Takacs, 1944), their reproduction being intimately related to the enzymic endowment of the host cell.

Thus an indirect approach to the problem of the enzymes utilized in virus synthesis is required. One possible mode of approach is to compare the metabolic activities, e.g., respiration or glycolysis of normal and virus-infected tissues (Racker and Kabat, 1942; Nickle and Kabat, 1944), but it has not so far provided significant results (Wood, Rusoff and Reiner, 1945; Utter, Reiner and Wood, 1945). On the other hand, study of individual enzyme systems in virus-infected tissues has given more hopeful results. Mouse-brains infected with the viruses of yellow fever, lymphocytic choriomeningitis or lymphogranuloma show increased dehydrogenase activity, due in part, it is claimed, to an increase in the activity of xanthine oxidase and pyruvic oxidase (Bauer, 1947). Also, chorioallantois and the chick embryo infected with yellow fever virus show increased xanthine oxidase activity (Bauer, 1948) due to increase in total xanthine-oxidase of these tissues and not to the mobilisation of enzyme from the rest of the egg. The significance of the increase in xanthine oxidase is obscure, since this enzyme is specific for the reactions: hypoxanthine$\longrightarrow$xanthine$\longrightarrow$uric acid, apart from its activity, as the Schardinger enzyme of milk, in oxidising many aldehydes to the corresponding acids; but pyruvic oxidase may play an important part in building up the reserve of adenosine triphosphate, an important source of energy.

A rather similar mode of approach to the problem is the study of the effects of enzyme inhibitors on the ability of viruses to multiply in their host cells; and the present paper describes results obtained when substances known to possess a varying range of inhibitory action on enzyme systems were tested for their effect on the reproduction of a bacterial virus and its host cell. Some preliminary results have already been published (Czekalowski and Dolby, 1949).

## METHODS.

### *Media.*

Two types of media were used for the growth of host cells and the preparation of phage suspensions:

a. *Simple media.*—Two simple media of the following composition were used:

| (1) | | (2)* | |
|---|---|---|---|
| $Na_2HPO_4$ | 6·0 | $K_2HPO_4$ | 0·7 |
| $KH_2PO_4$ | 3·0 | $KH_2PO_4$ | 0·3 |
| $MgSO_4$ | 0·2 | $MgSO_4$ | 0·01 |
| $NH_4Cl$ | 1·0 | $NH_4Cl$ | 1·0 |
| NaCl | 0·5 | $Na_2SO_4$ | 0·1 |
| Glucose | 4·0 | Sodium lactate | 10·0 |
| Dist. water | 1000 | Dist. water | 1000 |

* Friedlein (1928).

b. *Complex medium.*—This consisted of 2 per cent Difco Tryptose, 0·2 per cent glucose, 0·5 per cent NaCl and 0·01 M phosphate buffer, pH 7·2.

### *Diluting fluids.*

At first a 0·85 per cent saline solution containing 0·01 M phosphate buffer was used, but in further experiments the saline phosphate was supplemented with 0·005 M $MgCl_2$, in view of its stabilising effect on the T-series of phages (Adams, 1949).

### *Host cells and phages.*

The experimental systems were composed of *Bact. coli*, strain B (Cohen and Anderson, 1946), and T2 strain of phages (Anderson, Delbrück and Demerec, 1944). This system was chosen because both components can be readily prepared and accurately assayed.

In all experiments *Bact. coli* was grown in a water-bath at 37° for 150 min. to the approximate density of 2–5 $\times$ $10^7$ organisms per ml. In the complex medium any movement of the fluid was due to convection currents, but cultures in synthetic media were also aerated.

The number of phage particles added to the experimental systems was calculated to ensure infection of each bacterium by no more than a single lytic particle. In the preparation of phage-suspension the method of Cohen and Anderson (1946) was followed.

The population of *Bact. coli* was estimated by plating out suitably diluted samples of cultures in solid nutrient agar and counting the colonies after 48 hours' incubation at 37°. Phage assay was carried out by the method described elsewhere (Dolby and Czekalowski, 1949), based on the plaque-counting method of Delbrück and Luria (1942) modified by Hershey, Kalmanson and Bronfenbrenner (1943).

An estimate of the accuracy of the titration method may be obtained from the results of an experiment in which the effect of inhibitors on the phage alone was tested. None of the substances at the concentrations used produced any destructive effect on the phage, and the results of the experiment may be taken to show the normal range of variation in a titration of this sort (Table).

TABLE.—*Phage Titrations after Contact with Inhibitors.*

| Inhibitor. | Final concentration. | Plate counts: Plate 1. | Plate 2. | Average. | Population of phages in experimental systems after 20 minutes' contact with inhibitor. |
|---|---|---|---|---|---|
| Hydroquinone | $50 \times 10^{-3}$M | 234 | 229 | 232 | $2{\cdot}37 \times 10^{7}$ |
| Toluquinone | 5·5 „ | 236 | 201 | 219 | 2·23 „ |
| Tetramethyl-*p*-phenylene-diamine | 3 „ | 256 | 261 | 259 | 2·64 „ |
| Iodoacetic acid | 25 „ | 254 | 223 | 239 | 2·44 „ |
| Auramine | 0·66 „ | 229 | 263 | 246 | 2·51 „ |
| Urethane | 300 „ | 279 | 257 | 268 | 2·73 „ |
| Colchicine | 0·008 „ | 220 | 219 | 220 | 2·24 „ |
| Sodium cyanide | 50 „ | 218 | 207 | 213 | 2·17 „ |
| Sodium fluoride | 24 „ | 246 | 241 | 244 | 2·49 „ |

Control of phage suspension : $2{\cdot}14 \times 10^{7}$.
Final concentration of phages in experimental systems : $2{\cdot}88 \times 10^{7}$.
Average titre in experimental systems = 238.
Variation : + 12·6 per cent.
— 10·5 per cent.

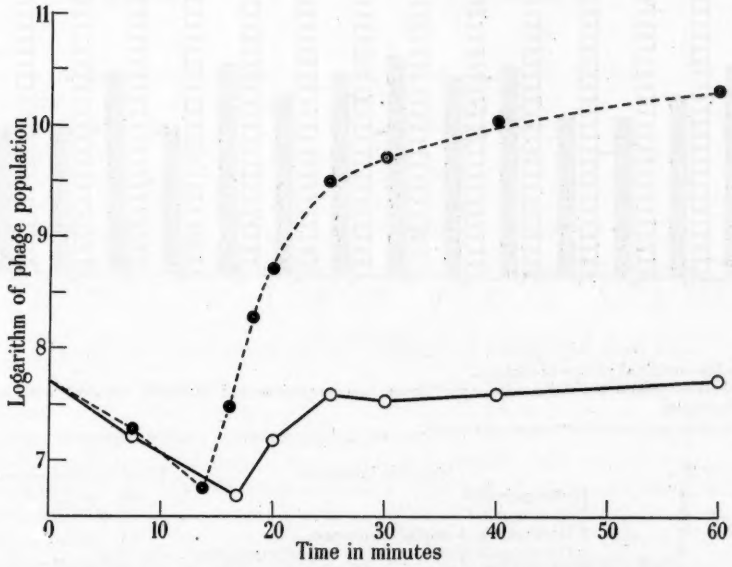


FIG. 1.—Adsorption and release of phages in the presence of $24 \times 10^{-3}$M sodium fluoride (continuous line) and in control (broken line).

*Experimental.*

The experiments performed were of two types :

1. Variations in the bacterial and phage counts during the adsorption and multiplication of the latter in the presence of inhibitors were followed by estimation of the bacterial and phage populations at different intervals. A bacterial culture of known density (about $5 \times 10^{7}$ organisms per ml.) was added to a solution of

the inhibitor at 37° and the addition of the phage suspension followed immediately. The course of the interaction was then estimated at intervals by taking two samples at the same time; one of these was assayed directly for its *Bact. coli* content, and the supernatant of the other, after centrifugation at 3500 r.p.m.

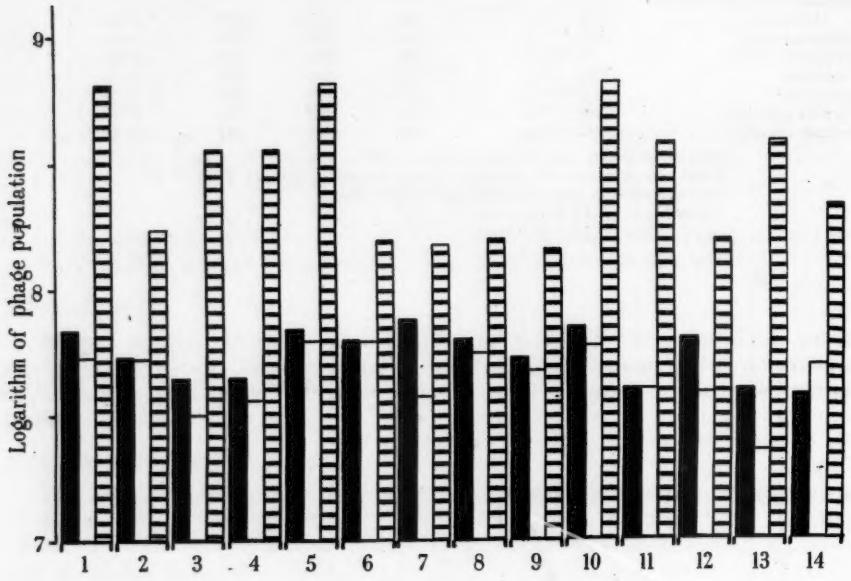


FIG. 2.

*Solid* blocks—original phage inoculum.
*Empty* blocks—phage particles recovered from the experimental systems treated with enzyme inhibitors.
*Shaded* blocks—controls (untreated systems).

| No. | Enzyme inhibitor. | Final concentration. |
|---|---|---|
| 1 | Hydroquinone | 50 × $10^{-3}$M |
| 2 | Toluquinone | 5·5 ,, |
| 3 | 2-Hydroxy-1, 4-naphthoquinone | 1 ,, |
| 4 | 2-Hydroxy-3-cyclohexyl-1, 4-naphthoquinone | 0·1 ,, |
| 5 | Tetramethyl-*p*-phenylenediamine | 3 ,, |
| 6 | 2, 4-Dinitrophenol | 0·2 ,, |
| 7 | Urethane | 300 ,, |
| 8 | Methionine | 10 ,, |
| 9 | Colchicine | 0·008 ,, |
| 10 | Auramine | 0·066 ,, |
| 11 | Mapharside | 0·04 ,, |
| 12 | Sodium selenite | 0·2 ,, |
| 13 | Cyanide | 50 ,, |
| 14 | Sodium fluoride | 24 ,, |

(The height of the centre column of No. 14 is incorrect. It should be of the same height as the column on the left.)

for 15 min. to deposit bacteria and adsorbed phages, was tested for phage (Dolby and Czekalowski, 1951) (Fig. 1).

2. Equal volumes of bacterial culture were added to two tubes containing equal amounts of inhibitor at 37°. The mixtures were incubated for 60 min., and then phage suspension added to one but not to the other. After a further 20 min. incubation the former was assayed for the phage and the latter for bacteria. In all cases controls, in which the inhibitor solution was replaced by water, were put up at the same time (Fig. 2).

## RESULTS.

The chemical compounds examined could be divided into five groups, according to their action on the experimental systems composed of host cells and bacterial virus.

GROUP I: *Substances which Affect neither Phage nor Host Cell.*

| Substance. | Final concentration. | |
|---|---|---|
| Benzedrine | 0·01, 0·1, 1 | × $10^{-3}$M |
| Caffeine | 0·5, 5 | |
| Malonates (acid or sodium salt) | 0·1, 0·5, 1, 5, 10, 15, 50, 100 | |
| Mapharside* | 0·004 | |
| Sodium fluoride | 1·25, 2·5, 5, 10 | |
| Sodium nitrite | 1, 10 | |
| Sodium succinate | 10, 50, 100 | |
| Stilboestrol | 0·01, 0·1, 1 | |
| Malachite green | 1 : 400,000, 1 : 500,000 | |

* Mapharside = Meta-amino-para-hydroxy-phenylarsine-oxide hydrochloride.

GROUP II: *Substances which Destroy both Phage and Host Cell.*

| Substance. | Final concentration. | |
|---|---|---|
| Acriflavine | 0·35, 0·7 | × $10^{-3}$M |
| 2-Hydroxy-3-cyclohexyl-1, 4-naphthoquinione | 1 | |
| Iodoacetic acid | 0·25, 1·35 | |
| Mapharside | 0·4 | |
| Sodium fluoride | 60 | |
| Sodium selenite | 20 | |
| Malachite green | 1 : 50,000, 1 : 100,000 | |
| Penicillin G | 500 and 1500 U/ml. | |

GROUP III: *Substances Exerting a Destructive Effect on Phages and a Static Effect on Host Cells.*

| Substance. | Final concentration. |
|---|---|
| Iodoacetic acid | 0·05 × $10^{-3}$M |
| Malachite green | 1 : 200,000 |

GROUP IV : *Substances Not Affecting the Reproduction of the Host Cells but having a Static Effect on the Phage.*

| Substance. | Final concentration. |
|---|---|
| Auramine | 0·66 |
| Colchicine | 0·008 |
| Cyanide | 50 |
| 2, 4-Dinitrophenol | 0·2, 1, 5 |
| Hydroquinone | 5, 7·5, 15, 30, 50 |
| 2-Hydroxy-1, 4-naphthoquinone | 0·01, 0·1, 1 |
| 2-Hydroxy-3-cyclohexyl-1, 4-naphthoquinone | 0·01, 0·1 |
| Mapharside | 0·04 |
| Methionine | 0·1, 1,10 |
| Sodium fluoride | 24 |
| Tetramethyl-*p*-phenylenediamine | 3 |
| Toluquinone | 5·5 |

× $10^{-3}$M

GROUP V : *Substances having a Destructive Effect on Phage but Not Influencing the Reproduction of the Host Cells.*

| Substance. | Final concentration. |
|---|---|
| Auramine | 0·066 |
| Cyanide | 1, 5 |
| Sodium selenite | 0·2, 2 |
| Tetramethyl-*p*-phenylenediamine | 0·3 |
| Urethane | 300 |

× $10^{-3}$M

Sodium mustard = (Di-(2-chloroethyl) methylamine hydrochloride) (previously studied by Herriot, 1949 and Herriot and Price, 1949) either destroyed *Bact. coli* cells ($10^{-3}$M) or prevented their reproduction ($10^{-4}$M). A similar effect was noticed with silver nitrate, which in final dilutions of 1 : $10^4$ and 1 : $10^5$ interfered with the host cells' reproduction. Inconclusive results were obtained with benzimidazole.

The substances of the last two groups (IV and V) share the property of not affecting the course of multiplication of the host cells. Some of them (tetramethyl-*p*-phenylenediamine, auramine, cyanide) at high concentrations did not destroy the phages, but prevented their multiplication, so that only the original inoculum was recovered; in lower concentrations, however, the phage was obviously destroyed. A possible explanation for this paradoxical phenomenon may be given in the following terms: High concentrations of these substances affect the enzyme system of the host cell required for phage reproduction to such an extent that this system is no longer capable of combining with phage particles, as a result of which the phage, after the initial adsorption, is released in unaltered form and can be assayed. Lower concentrations of these substances, on the other hand, may only modify the appropriate enzyme or receptor to such an extent that it retains the property of binding the phage particle, whilst its link with other biological systems involved in bacterial virus reproduction becomes severed as a result of which the adsorbed phage is no longer reproducible.

Mapharside in concentrations higher than $4 \times 10^{-5}$M affects both elements of the experimental system; in lower concentrations it affects neither.

Quinones, within the range of the concentrations examined, prevented the multiplication of phage, but did not affect the reproduction of host cells. The only exception was the 2-hydroxy-3-cyclohexyl-1, 4-naphthoquinone, which at $10^{-3}$M concentration inhibited the multiplication of both host cells and phages.

The results of experiments on enzyme inhibitors are often presented in terms of the percentage inhibition in comparison with controls containing no inhibitor. Ackerman and Potter (1949) discuss this point at length and stress that this approach may lead to incorrect conclusions because, with some inhibitors, the percentage inhibition is a function of the enzyme concentration. The evidence from our experiments on the effect of substances belonging to Groups IV and V on phage multiplication has therefore been summarized in the form of a histogram (Fig. 2), in which the numbers of lytic particles both in the experimental systems and in controls are expressed as their logarithms.

## DISCUSSION.

The substances which, in this work, have been found to interfere with the multiplication of bacterial virus but not to influence the reproduction of their host cells belong to many different chemical groups, but they have in common the property of interfering with those enzymes which work in conjunction with the cytochrome system, and particularly that of interfering with the succinoxidase system. It is known that inhibitors of this system may act either specifically, by inhibiting only one of its components, and so interrupting the chain of electron carriers, or non-specifically on the whole system, by disturbing the mutual spatial relationship of its components (Keilin and Hartree, 1940 and 1949). Most of the substances investigated by us belong to the former group.

Of the substances known to inhibit the group of enzymes containing sulphydryl groups, to which succinic dehydrogenase belongs, we have found urethane (Keilin and Hartree, 1939), selenite (Barron and Kalnitzky, 1947) and mapharside (meta-amino-para-hydroxy-phenylarsine-oxide hydrochloride) to inhibit virus growth; but malonic acid, which is the most specific inhibitor of succinic dehydrogenase, did not. Other substances which may fall into this group are: Auramine (Quastel and Wheatley, 1931 b); Colchicine (though it has also been suggested that other enzymes may be affected by this substance) (Cook, Krake, McDevitt and Barlett, 1946; Dustin, 1947; Dustin and Lecomte, 1949; MacFarlane and Schmock, 1948); and tetramethyl-*p*-phenylenediamine, since it has been established that oxidation products of some aromatic diamines inactivate enzymes of the sulphydryl type within the cell (Kensler and Rhoads, 1945).

The *p*-phenylenediamine may also, however, act on one of the components of the cytochrome system, which completes the transfer of hydrogen from the succinic dehydrogenase to molecular oxygen; for it has been shown to inhibit the "respiratory catalyst" which acts as link between cytochrome c and b, a property which it has in common with other substances, such as BAL, hydroquinone and phenylurethane (Slater, 1949 and 1950). The naphthoquinones also act on some factor, which is neither cytochrome c nor b, but nevertheless prevents the interaction of these two elements of the respiratory system (Ball, Anfinsen and Cooper, 1947). Other inhibitors which act on different parts of

this system are : cyanide, which inhibits cytochrome oxidase (Keilin and Hartree, 1939 ; Stannard and Horecker, 1948), and fluoride, which probably combines with the cytochromes, since they are haematin compounds (Slater, 1949).

The mode of action of methionine and 2-4-dinitrophenol is unknown. The experiments of Cohen and Fowler (1948) and of Fowler and Cohen (1948) show clearly, however, that methionine exerts an inhibitory effect on multiplication of phages by delaying synthesis of desoxyribonucleic acid. The effect of 2-4-dinitrophenol is probably non-specific since it decreases the rate of oxidation of many carbohydrates, although succinate and fumarate are affected to the greatest extent (Clifton and Logan, 1939). It inhibits also the synthesis of adaptive enzymes (Reiner, 1946).

Comparison between the cytochrome systems of mammalian cells and of micro-organisms is difficult because spectrographic analysis shows that there are qualitative differences between them : one or more of the components present in mammalian cells are missing from the group of facultative anaerobic bacteria, to which *Bact. coli* belongs. Thus, whilst it has been established beyond doubt that *Bact. coli* grown aerobically possesses some cytochromes, these differ from their analogues in the mammalian cytochrome system, the bands of cytochromes a and b being replaced by others known as $a_2$ (an autoxidisable component fulfilling the function of cytochrome oxidase) and $b_1$ respectively, while the bands for cytochromes c and $a_3$ are missing (Stephenson, 1949). How closely the two systems are related is not yet clear, but it seems reasonable to suppose that the main difference in their function probably lies in the interaction of cytochromes $b_1$ and $a_2$ in *Bact. coli* cells in the absence of cytochrome c.

It is obvious, therefore, that the results obtained in our experiments cannot be interpreted in the light of data accumulated for the cytochrome systems of mammalian cells. This is confirmed by the fact that malonate, which strongly inhibits mammalian succinic dehydrogenase, and cyanide, which inhibits mammalian cytochrome oxidase, did not inhibit the growth of *Bact. coli* in quite high concentrations, although the latter did inhibit phage multiplication.

## SUMMARY.

An indirect approach to the problem of the enzymes involved in bacterial virus synthesis was attempted, by studying the effect of enzyme inhibitors on the multiplication of phage (T2) and its host cell (*Bact. coli*, strain " B ").

The substances examined are divided into five groups according to their action on the host cell and the bacterial virus. Two groups of these substances are discussed in view of a finding that, while not affecting the reproduction of the host cells they exert either a static or destructive effect on the bacterial virus.

These substances belong to many different chemical groups but they share the property of interfering with those enzymes which make use of the cytochrome system in mammalian cells or with the cytochromes themselves, and particularly that of interfering with the succinoxidase system.

Qualitative differences between the cytochrome systems of mammalian cells and of micro-organisms must be remembered in interpreting these results.

The author is very much obliged to Dr. D. E. Dolby (now at the Lister Institute, Elstree), who participated in these experiments. My grateful thanks are

due to Professor Louis Fieser, Department of Chemistry, Harvard University, Mass., for his most generous gift of naphthoquinones used in these experiments; also to Mr. A. Myers for technical help.

Since this paper was submitted for publication some new information has been obtained by Foster and Johnson (R. A. C. Foster and F. H. Johnson, *J. Gen. Physiol.*, 1951, **34**, 529–550) about the action of urethane on *Bact. coli* strain B and the synthesis of T2-phage.

These authors showed that under certain experimental conditions the phage-infected cell passes through some phases characterised by the differences in their sensitivity to urethane. The synthesis of desoxyribonucleic acid of T2-phage takes place during the phase of complete urethane sensitivity.

The hypothesis offered suggests that urethane does not prevent the release of the templates essential for bacterial-virus DNA-synthesis, but acts upon the synthetic processes in which they are concerned.

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# SERUM NEPHRITIS IN RABBITS.

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ATTEMPTS to produce experimental nephritis date from the beginning of the century. With the exception of chemical nephritis, the early experiments, based on the production of a sensitivity to bacteria or foreign protein, were failures. Such successes as were claimed have all been shown to be due to confusion with the "spontaneous nephritis" found in some rabbits (Bloomfield, 1919; Bell and Hartzell, 1919). The first successes were reported by Masugi (1934) and Masugi and Sato (1934), using a nephrotoxic serum (anti-rabbit-kidney serum) or horse serum. In 1943 Rich and Gregory recorded an acute diffuse glomerulonephritis in rabbits sensitised to horse serum, and more recently Hawn and Janeway (1947) produced glomerulonephritis in rabbits by injecting bovine gamma globulin. In these groups of experiments relatively large quantities of antigen were used over a comparatively short time. A different technique has recently been published by McLean, Fitzgerald, Younghusband and Hamilton (1951), in which glomerulonephritis in rabbits was produced by daily injection of small amounts of horse serum over periods up to thirteen months.

In the experiments described in this paper glomerulonephritis in rabbits has been produced by the weekly injection of horse serum up to periods of seven months. While the number of animals used is too small to allow of any statistical analysis of the results, effects have been observed which enable the evolution of the disease to be followed histologically; they also show the relation between the onset of the nephritis and the state of sensitivity of the animal. The relation between human and experimental nephritis has also been considered. Previous authors have likened experimental serum nephritis to human acute nephritis, and have in consequence concluded that the human disease is an allergic reaction. In the experimental nephritis described here we have noted several differences, and have attempted to show that the renal lesions are identical with certain manifestations of human periarteritis nodosa which have been reported in the literature. Human acute nephritis may be an allergic disease, but if the experimental work on which the analogy has been based is open to a different interpretation then the analogy has been falsely drawn.

## METHODS.

Thirty cross-bred rabbits of both sexes, 3–6 months old were used. They were separately caged and fed on oats, bran mash and greenstuffs in season. The experiments were conducted in three groups over a period of 2 years. One group was completely finished before the next was started; this procedure was used to

minimise the effect of external influences on the results which are all considered together.

Burroughs Wellcome horse serum No. 2 without preservative was used as the antigen throughout, and was injected intravenously. Each rabbit received an initial injection of 20 ml. On the 12th day an intradermal test using 0·2 ml. serum was performed to test sensitivity. On the 13th day 1·0 ml. serum was injected intravenously, and thereafter weekly injections were given. The amount of these later doses varied with the reaction of the rabbit. It was generally found that for the first 90–120 days the dose had to be kept between 1·0 and 5·0 ml.; larger doses produced anaphylactic shock and death. As will be seen in the tabulated results, about half of the animals did die before the 90th day after exhibiting manifestations of shock. After 120 days the amount of serum could be rapidly increased and up to 20 ml. were given weekly. During this period all except 4 rabbits sickened and died; no injections were given to these 4 animals after 7 months and they were killed after 10 months.

The clinical condition of all animals was recorded and blood urea estimations were made on some of them.

At post-mortem sections of appropriate organs were fixed in formol saline and Zenker. Sections were stained with haematoxylin and eosin and van Gieson.

## RESULTS.

The results are shown in the table, which includes the time of survival, the number of injections and total amount of horse serum given, the mode of death and the naked-eye and histological appearances of the kidneys.

Skin sensitivity tests were carried out on the first two groups of rabbits, eighteen in number, at monthly intervals. It is interesting that all the tests between the 30th and 120th days gave an immediate positive reaction (24 hours) with erythema and oedema, and at 7 days had produced a typical Arthus reaction with haemorrhage and necrosis. All the animals tested before 30 days and after 120 days gave the immediate positive response but failed to develop the necrotic lesion.

Clinically all the animals manifested some signs of anaphylactic shock in the early days of the experiments, and as a result 16 of them died during the first four months. This anaphylactic death occurred in two forms, acute with death in convulsions during or within a few minutes of the injection, and delayed. In the delayed type the animal showed signs of shock during the injection but apparently recovered; however, within 24 hours sudden collapse was noted, respiration ceased, the heart began to beat irregularly and the animal died. Up till the time of death the general health of these animals was excellent.

After 120 days, when no more signs of anaphylaxis were noted, the animals continued well until the final illness began. This first showed itself with loss of appetite and some loss of weight. The animals then became listless and lay in the corner of the cage, eating less and less and becoming progressively thinner till eventually coma and death ensued, the whole illness lasting from 10–70 days.

Four animals survived in good health and were killed after 318 days.

## HISTOLOGICAL FINDINGS.

In those animals which developed glomerulotubal nephritis the kidneys were twice the normal size and appeared pale. On section the renal substance looked oedematous but the normal radial striation was unimpaired. Histologically few

TABLE.—*Detail of Injections, Survival, Mode of Death and Renal Lesions.*

| Animal. | Number of injections. | Amount of serum in ml. | Survival in days. | Mode of death.* | Renal lesions. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Tuft. | | Crescent. | | Tubular degeneration. | Cellular infiltration. |
| | | | | | Hyalinisation. | Adhesions. | Exudate. | Proliferation. | | |
| R. 39 | 3 | 23 | 14 | An | 0 | 0 | 0 | 0 | 0 | 0 |
| R. 17 | 2 | 22 | 16 | „ | + | + | 0 | 0 | 0 | 0 |
| R. 18 | 3 | 25 | 22 | „ | 0 | + | 0 | 0 | 0 | 0 |
| R. 28 | 4 | 32 | 25 | „ | + | + | 0 | + | + | + |
| R. 25 | 3 | 12 | 25 | „ | 0 | 0 | 0 | 0 | 0 | 0 |
| R. 10 | 4 | 14 | 36 | „ | 0 | 0 | 0 | 0 | 0 | 0 |
| R. 9 | 4 | 12·5 | 37 | „ | + | 0 | 0 | 0 | 0 | 0 |
| R. 19 | 5 | 14 | 42 | „ | ++ | 0 | 0 | 0 | + | + |
| R. 20 | 5 | 11 | 42 | „ | 0 | 0 | 0 | 0 | 0 | 0 |
| R. 47 | 7 | 43 | 46 | „ | 0 | 0 | 0 | 0 | 0 | 0 |
| R. 30 | 8 | 61 | 52 | „ | 0 | 0 | 0 | 0 | 0 | 0 |
| R. 8 | 7 | 26 | 58 | „ | 0 | 0 | 0 | 0 | + | + |
| R. 29 | 10 | 74 | 74 | „ | 0 | 0 | 0 | 0 | 0 | 0 |
| R. 48 | 11 | 49·5 | 74 | „ | + | 0 | 0 | 0 | 0 | 0 |
| R. 26 | 14 | 153 | 103 | „ | + | 0 | 0 | 0 | 0 | 0 |
| R. 43 | 18 | 68 | 124 | „ | 0 | 0 | 0 | 0 | 0 | 0 |
| R. 54 | 17 | 137 | 126 | ? | ++ | + | + | 0 | 0 | + |
| R. 50 | 19 | 164 | 135 | Coma | ++ | 0 | + | + | + | + |
| R. 52 | 20 | 170 | 138 | „ | + | 0 | + | + | + | + |
| R. 7 | 19 | 111 | 145 | „ | ++ | 0 | + | + | + | + |
| R. 12 | 21 | 141 | 158 | „ | ++ | 0 | + | + | + | + |
| R. 51 | 22 | 226 | 161 | „ | + | 0 | + | 0 | + | + |
| R. 16 | 25 | 247 | 183 | „ | ++ | + | 0 | + | + | + |
| R. 15 | 26 | 272 | 189 | „ | + | 0 | 0 | 0 | 0 | 0 |
| R. 11 | 30 | 238 | 206 | „ | ++ | 0 | + | + | + | + |
| R. 53 | 27 | 324 | 220 | „ | ++ | 0 | + | + | + | + |
| R. 44 | 27 | 318 | 318 | Killed | + | + | 0 | ± | 0 | 0 |
| R. 45 | 27 | 320 | 318 | „ | + | + | 0 | 0 | 0 | 0 |
| R. 46 | 27 | 358 | 318 | „ | 0 | 0 | 0 | 0 | 0 | 0 |
| R. 49 | 27 | 325 | 318 | „ | + | 0 | 0 | 0 | 0 | 0 |

* An = anaphylactic death either acute within a few minutes of injection or delayed occurring within 24 hours after exhibiting symptoms of anaphylactic shock.

or no normal glomeruli were seen (Fig. 1*a*). The tufts were enlarged and relatively bloodless, the capillaries being narrowed by an eosinophil hyaline thickening of their basement membranes and some proliferation of their endothelium. The normally scanty interstitial connective tissue of the tufts also showed a marked hyalinosis. Sometimes this hyalinosis was accompanied by karyorrhexis of the nuclei in the tuft, producing an appearance of necrosis apparently due to degeneration of the capillary endothelium from pressure of the swollen basement membrane and interstitial tissue. In many of the capsular spaces there was a strongly eosinophilic exudate in which were groups of red blood corpuscles. The capsular epithelium showed proliferation with numerous crescents, which had progressed in places to fill completely the capsular space leaving no trace of the capillary tuft.

The tubular changes were equally well marked. There was interstitial oedema in the region of the 1st convoluted tubule but no marked epithelial changes. The 2nd convuluted tubules were dilated, contained eosinophil hyaline casts similar in structure to the capsular exudate and showed marked flattening of their epithelium.

No changes could be detected in the afferent arterioles of the glomeruli and the interlobular vessels also appeared healthy.

Cellular infiltration of varying degree was found in all the affected kidneys. It was either diffuse or arranged concentrically round the glomeruli, throwing them into relief. Nearly all the cells were histiocytic in type with a slightly basophil cytoplasm, some showing the characteristic cart-wheel nucleus of the plasma cell. Some eosinophils were demonstrable in nearly all cases, but very few polymorphs or lymphocytes were seen. It was noticeable that there appeared to be no invasion of the capillary tuft, although large groups of cells were often found in the neighbourhood of the vascular pole.

In the van Gieson stained sections early fibrosis was noticeable in the interstitial tissue and glomeruli, but no real scarring was evident (Fig. 1*b*).

In all 10 rabbits showed this complete picture of glomerulotubal nephritis, and we have thought it possible from these and the other rabbits less severely affected to trace the sequence of changes which take place.

It appears that the first change in the tuft is the thickening and hyalinosis of the capillary basement membrane and the interstitial tissue (Fig. 1*d*). This may be the only change, as it was found in 6 animals dying before the 120th day and in 3 of the 4 surviving after 7 months of injections. When this change is more severe karyorrhexis of the nuclei may be seen; the capillary lumina become narrowed and the tuft becomes permeable with a resulting exudate of red cells and plasma into the capsular space (Fig. 2*a*, 2*b*). If healing takes place at this stage a rather enlarged rigid tuft is left which may be adherent, with some capsular fibrosis. Continuation of the process results in proliferation of the capsular epithelium and the formation of crescents which may eventually completely fill the capsular space (Fig. 2*c* and 2*d*).

The tubular changes do not appear to be entirely secondary to destruction of the glomeruli, as in rabbit No. 8 they were found without any glomerular abnormality, and in rabbit No. 19 which showed only the early changes in the tuft. In the animals showing the fully developed nephritis the degree of tubular change was commensurate with the degree of glomerular change, and it seems reasonable to suppose that the disease process affects the nephron as a whole, producing some changes which appear side by side.

Another histological abnormality noted was the occlusion of capillaries of the tuft by hyaline thrombi. As will be seen from the table, this was an almost constant finding in animals dying an anaphylactic death after the first month. This change was not confined to the kidneys, and could be demonstrated in most organs sectioned, particularly the lungs and liver. This hyaline plugging of capillaries in rabbits in anaphylactic shock has been noted by several experimenters (Bohmig, 1934; Ahlstrom, 1936; Gregory and Rich, 1946) who have discussed its possible rôle in the experimental allergic conditions they described. No evidence was found in our experiments that it played any part in the production of nephritis.

In 19 of the animals histological evidence of active, healing or healed periarteritis nodosa was found. In 14 this was confined to the circumflex coronary vessels, and in the remaining 5 animals respectively to the circumflex coronary and renal pelvic vessels, the circumflex coronary vessels and skeletal muscle, the renal pelvic arteries, the pulmonary arterioles and skeletal muscle.

Only one instance of the well-known "spontaneous nephritis" of rabbits was observed in this series although we have seen it in several other animals. The lesions cannot be confused with the true experimental nephritis, as they occur locally in wedge-shaped areas, and consist of interstitial fibrosis with some tubular atrophy (not the dilatation seen in true degeneration) and some fibrosis of the glomerular capsule. The tuft is small but histologically normal, and the cellular reaction, if any, is lymphocytic.

Blood urea estimations were performed on six of the last group of rabbits. Two of these died in coma, R. 51 and R. 53, and the terminal blood ureas were 161 and 400 mg. per 100 ml. R. 53 had shown a steady rise from the 156th day till death on the 220th day. The remaining animals on which the test was performed, R. 44, R. 45, R. 46 and R. 49, were the four animals surviving the experiment and were killed on the 318th day. The results varied between 28 and 50 mg. per 100 ml., but consecutive estimations on individual animals showed no progressive increase.

## DISCUSSION.

### *Relation of Sensitivity to Onset of Nephritis.*

The number of animals is too small and the scope of the investigation too narrow to allow of any valid statistical inquiry into the possible allergic nature of the induced nephritis. All the animals, however, became sensitive to horse serum as evidenced by the production of a positive skin test with an accompanying Arthus phenomenon. This phase lasted till 90–120 days, and the weekly dose of serum had to be regulated carefully to avoid fatal anaphylaxis, which, nevertheless, occurred in 15 of the 30 animals. The full picture of glomerulonephritis did not develop in any of these 15 animals, although early changes in the capillary tuft or tubules were seen in seven of them (R. 17, R. 28, R. 9, R. 19, R. 8, R. 48 and R. 26). After 120 days the sensitivity of the rabbits changed, so that while the intradermal inoculation of the antigen produced erythema and oedema in 24 hours the lesion did not progress to necrosis (that is, the Arthus phenomenon did not develop), and large weekly doses of serum could be given intravenously without inducing anaphylaxis. It was during this period of altered sensitivity that glomerulonephritis developed, and of the 14 animals concerned 10 died in

apparent uraemic coma and 4 survived. Three of these survivors showed hyalinisation of the tuft with capsular adhesions but no crescent formation or tubular change.

McLean *et al.* (1951) have produced the same glomerulonephritis in rabbits using small doses of horse serum given intravenously daily for periods from 3–13 months. They comment that the time of development of the nephritis is unpredictable, as it appeared at all stages of their experiments after the first 3 months. They noted that skin tests were positive in all but one of their animals showing nephritis, but they do not record the qualitative difference in the reaction which we have noted. Rich and Gregory (1943) induced nephritis in 7 out of 9 rabbits sensitised to horse serum, given one or two further injections and killed on or before the 26th day. All the animals showed positive skin tests on the 12th day of the experiment, and some progressed to the haemorrhage and necrosis of the Arthus phenomenon. Hawn and Janeway (1947), using a single large dose of bovine serum, noted no evidence of acute nephritis, and only 1 out of 18 rabbits showed a picture described as healed nephritis.

These results show that repeated injections with the production of sensitivity to the antigen are required to produce the nephritis ; single large injections do not. As to the time of onset and state of sensitivity when the nephritis develops the results quoted appear confusing. Our own findings, however, were quite clear cut, and a closer analysis of the other reported experiments seems to offer a possible explanation.

The skin tests as we performed them gave an immediate positive reaction throughout the experiment. The Arthus phenomenon, however, was only observed between the 30th and 120th days. The full picture of acute nephritis only developed in our animals when the Arthus phenomenon had disappeared, and the earlier changes of nephritis occurring before the 120th day were all (with one exception, R. 8) found in animals dying before the 42nd day, and probably had begun before the 30th day when the Arthus phenomenon became positive. If it is assumed, then, that nephritis does not develop during that stage of sensitivity when the Arthus reaction is positive, the other reports in the literature can be fitted into the picture. As has been said, McLean *et al.* (1951) did not note any qualitative difference in their skin sensitivity tests, but noted the onset of nephritis at 90 days in 3 out of 8 animals, at 90–120 days in 7 out of 8 animals and at longer periods up to 13 months in 10 out of 15 animals ; that is, the maximum incidence is at the time we have recorded in our own experiments. In the experiments of Rich and Gregory the nephritis had developed before the 30th day, and although they state that some animals showed a positive Arthus reaction they do not say which, and it may have been that those which did failed to develop nephritis.

### *The Nature of the Antigen.*

The antigen used in these experiments and those of some other observers mentioned has been whole horse serum. Other experiments have been conducted with whole bovine serum or various equine and bovine serum fractions. Hawn and Janeway (1947) found that with bovine serum albumen the lesions were confined to arteries and were less severe than those caused by whole serum. Bovine gamma globulin produced a striking acute nephritis with few and insignificant changes in the arteries of the kidney and elsewhere. Whole bovine serum pro-

duced a widely dispersed acute arteritis, and the authors comment on the similarity of the lesions as a whole to those given by horse serum.

That gamma globulin is the effective fraction of serum in the production of nephritis has also been reported by More and Waugh (1949), although More and Kobernick (1951), using whole horse serum and streptococci and bovine gamma globulin and streptococci as antigens, found the incidence of acute glomerulonephritis the same in each case. Commercial horse serum contains between 5 and 7 g. per 100 ml. protein, the albumen varying between 1 and 3 g. and the globulin between 3·5 and 4·0 g. per 100 ml. Globulin then forms the major part of the protein of horse serum, and the use of whole serum for producing experimental nephritis should be little or no less efficient than the separated globulin fraction providing adequate amounts are used.

Wissler, Smull and Lesh (1949) also studied the effects of various horse serum fractions in producing renal lesions in rabbits. Their conclusions are that glomerulitis of an acute necrotizing type was seen in some rabbits without particular correlation to the fraction injected, and that gamma globulin produced little reaction histologically.

The apparent contradictions of these published results may be due to the short duration of the experiments. In all cases the animals were sacrificed within a month and, as has been pointed out, this is apparently not the optimum time for development of nephritis, although the stage of sensitivity is such that it may develop.

### *Nature of the Lesions.*

We have already indicated the probable line of development of lesions as—

1. Hyalinisation of the non-cellular components of the capillary tufts with resulting increased permeability. There also occurs some proliferation of the cells of the tuft.
2. Exudate of serum and red cells into the capsular space leading to proliferation of the capsular epithelium.
3. Obliteration of the space and tuft by fibrosis.
4. Concurrent hyalinisation of the non-cellular components of the interstitial tissue of the cortical tubules, eventually progressing to fibrosis. The tubules become dilated and their epithelium atrophic.
5. Cellular infiltration of the cortex by histiocytes at all stages of the disease.
6. Complete absence of arterial and arteriolar changes.

The primary change of a hyaline degeneration of the acellular structures of the tuft capillaries is similar to that described in the group of disorders collectively and loosely described as " vascular collagen disease," which includes such diseases as periarteritis nodosa, disseminated lupus erythematosus and dermatomyositis. The varying histological appearances of these diseases is the result of a basic process of " hyaline " or " fibrinoid " degeneration affecting vessels of different structure, and the resultant healing and fibrosis produce changes which differ with the organ affected and the severity of the initial lesion. In the kidney the process we have observed produces a picture of glomerulotubal nephritis akin to that seen in the acute nephritis of humans, and McLean *et al.* (1951) have noted the similarity of their experimental lesions to the human disease. There are,

however, several differences which may indicate that this similarity is only in appearance, while actually the process is basically different.

In human acute nephritis the cellular proliferation of the tuft is more marked, and there is an infiltration of the tuft with polymorphonuclear leucocytes. In the rabbit cellular invasion of the tuft does not occur unless there is " necrosis " of the capillary endothelium ; even then the invasion is minimal and consists of a few histiocytes or plasma cells. The histiocytic reaction seen generally in the cortex in the serum nephritis is not a recognised feature of human nephritis. The reaction of the capsular epithelium to haemorrhage and protein exudation is the same in both diseases as is the resulting obliteration of the glomerulus.

The most striking difference between the two diseases is the absence of any change in the arterioles and arteries in the rabbit serum nephritis. In the human disease in the acute stage the ischaemia of the tuft leads to a compensatory hypertension, which in turn is said to cause fibrinoid necrosis of the afferent arteriole, producing greater ischaemia of the tuft and more hypertension, with the establishment of a vicious circle and eventual destruction of the kidney. In the early stages of nephritis in man arteriolar damage is uncommon, but later when crescent formation and glomerular obliteration are seen arteriolar and arterial change are nearly always present. This is not so in the rabbit nephritis, where no lesions of the renal arterial tree attributable to the glomerular lesions were seen at any time. It might be argued that despite the advanced histological picture the development of the disease was so rapid that insufficient time had elapsed for the arteriolar changes to manifest themselves. In the ten animals concerned the period over which they sickened and died ranged from 10 to 70 days, five of them being 28 days or more ; some of these should have developed arteriolar damage, presumably having had glomerular ischaemia from 1 to over 2 months.

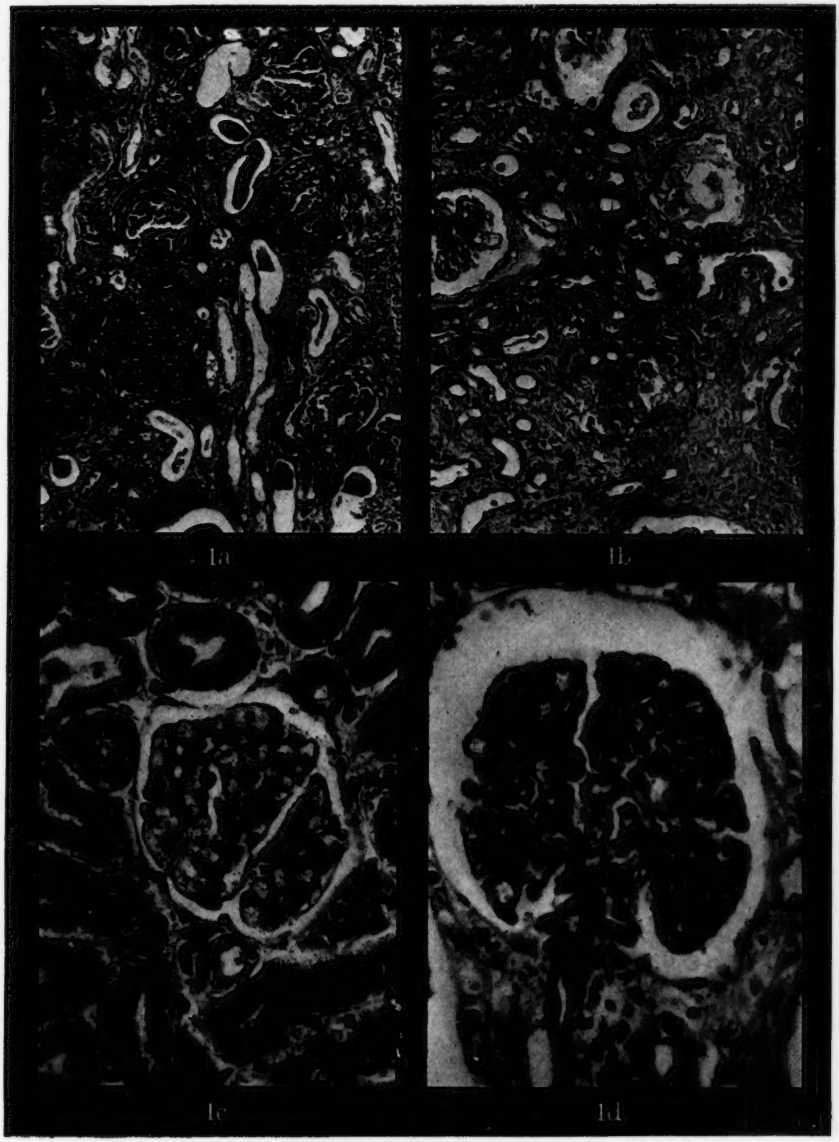
Although most of the experimental work showing that arteriolar necrosis in the kidney follows hypertension has been done on rats, Wilson and Pickering (1937–38) have shown that similar changes occur in the rabbit. The absence of these changes in the serum nephritis described here is not therefore due to the animal concerned, and it is possible that this particular type of glomerular damage does not lead to hypertension.

Recently Davson, Ball and Platt (1948) and Wainwright and Davson (1950) have studied the kidney in 70 cases of human periarteritis nodosa, and have separated a group of 13 cases characterised by a widespread necrotising glomerulitis without hypertension, with death due to renal failure. The histological picture of the glomerulitis is similar to our own, their fibrinoid necrosis corresponding in appearance to the hyalinisation in the rabbit kidney. Capsular

EXPLANATION OF PLATES.

FIG. 1.—Stages in the development of serum nephritis.
*a*. Glomerular exudate, capsular proliferation, tubular degeneration and periglomerular histiocytic infiltration. R. 50. × 166.
*b*. Interstitial fibrosis of cortex. R. 53. × 166.
*c*. Normal glomerulus. × 450.
*d*. Hyalinisation of basement membrane of capillaries of the tuft. R. 11. × 450.

FIG. 2.—Stages in the development of serum nephritis.
*a*. Commencing haemorrhage in capsular exudate. R. 51. × 450.
*b*. Capsular exudate and commencing capsular proliferation. R. 12. × 450.
*c*. Necrosis of hyalinised tuft and capsular proliferation. R. 51. × 450.
*d*. Complete replacement of tuft by hyperplastic capsular epithelium. R. 50. × 450.



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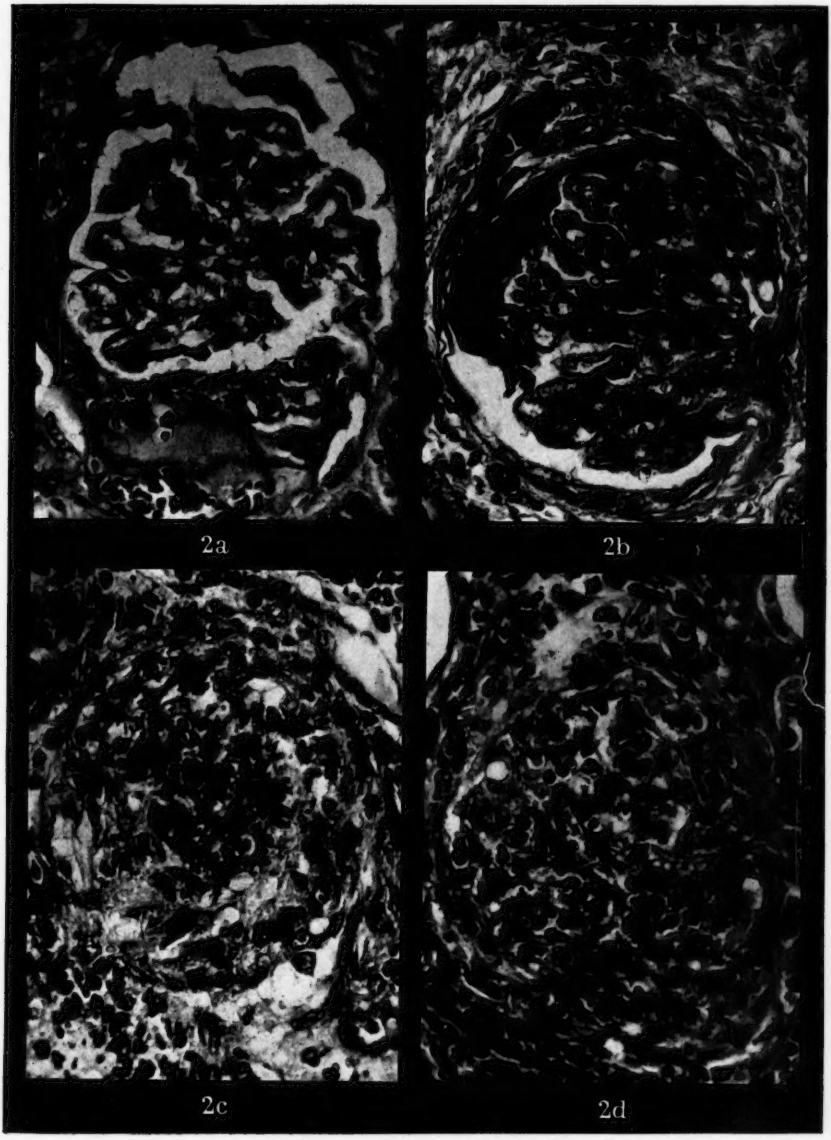

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exudate, crescent formation and periglomerular inflammatory cell infiltration were also described. The tubular changes also correspond exactly, and apart from two of their cases no afferent arteriolar change was seen. We have recently seen a post-mortem on a woman aged 57 who clinically was thought to have staphylococcal pneumonia. Sections showed the lung condition to be an acute periarteritis of the pulmonary arterioles. The kidneys showed a glomerular fibrinoid necrosis with a marked histiocytic and eosinophilic cellular infiltration, particularly in the periglomerular region. There were no hypertensive changes in the kidney arteries or arterioles. Sections of the spleen also showed an acute periarteritis of the arterioles and smaller arteries. The basic pathology of this case seems to be the same as that of the experimental lesions we have described, and would appear to be identical with the glomerular changes described by Davson, Wainwright and their co-workers.

Following the work of Rich and Gregory (1943) sensitisation with horse serum is now a standard method of producing lesions of periarteritis nodosa in the rabbit, and we have described such lesions occurring particularly in the circumflex coronary vessels of two-thirds of our animals. It seems to us therefore that the experimental serum nephritis described here and by other authors is a manifestation of the glomerulitis which sometimes accompanies periarteritis nodosa, and is not strictly comparable to human acute nephritis.

SUMMARY.

A glomerulotubal nephritis induced in rabbits with horse serum is described.

The relation of the type of sensitivity and the onset of nephritis is discussed.

It is suggested that this type of experimental nephritis is a manifestation of periarteritis nodosa, and is identical with a similar form of the disease occurring in man.

Our thanks are due to Mr. J. Johnstone for the preparation of the histological material, and Miss M. H. Shaw for help with the microphotography.

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# THE EFFECT OF CORTISONE ON THE REACTION TO QUARTZ IN THE PERITONEAL CAVITY.

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CORTISONE is known to affect the rate of healing of wounds by retarding the proliferation of new blood-vessels and by diminishing the amount of collagen formed. The present experiments were designed to determine whether cortisone has a similar inhibitory effect on the fibrosis in chronic inflammation.

Finely powdered quartz was selected as the agent for producing chronic inflammation, as the results could be expected to be more consistent than if a bacterial infection were employed. The quartz was given intraperitoneally, this being a simple and reliable method of producing silicotic fibrosis. By these experiments we hoped to determine not only the possible effects of cortisone on silicosis in particular, but also perhaps to learn something of the fundamental effects of cortisone on the development of granulation tissue in general. The results show that with the dosage used, cortisone suppresses the fibrosis in mice, retards it in rabbits and to a less extent in rats, and has no appreciable effect in guinea-pigs.

## METHOD.

Rabbits, guinea-pigs, rats and mice were used, and the procedure of introducing quartz into the peritoneal cavity was based on the technique of Miller, Sayers and Yant (1934).

Powdered quartz (Snowit II prepared from a sand) with a particle size of less than 2μ was made up as a 10 per cent suspension in saline and sterilized by autoclaving. It was then agitated with glass beads in a Kahn shaker for about an hour to break up any aggregations and produce a homogeneous suspension. A single measured dose of this was given intraperitoneally to each animal. In rabbits and rats it was inserted through a laparotomy under ether anaesthesia, the wound subsequently being sutured in layers. In guinea-pigs and mice it was given by simple intraperitoneal injection without anaesthetic.

Half the number of animals of each species were given subcutaneous injections of cortisone acetate daily (Table), commencing on the day the quartz suspension

TABLE.—*Dosage of Cortisone Related to Body Weight.*

| | Daily dose of cortisone (mg.). | Average weight (g.). | Dose per kilo (mg.). |
|---|---|---|---|
| Rabbit | 20 | 2500 | 8 |
| Guinea-pig | 10 | 600 | 16·5 |
| Rat | 5 | 175 | 28·5 |
| Mouse | 2·5 | 20 | 125 |

was administered. The remainder were used as controls, and given daily injections of corresponding volumes of the aqueous suspending vehicle employed in the particular cortisone preparation used.

Pairs of animals consisting of a cortisone-treated one and a control of the same species were killed at varying intervals, and when an animal died the corresponding animal of the pair was killed. The intention was to extend the experiment to 90 days, but only in the case of the rabbit was this achieved, the other species dying before this period had elapsed.

## RESULTS.

*Mice.*—Following intraperitoneal inoculation of 0·3 ml. of 10 per cent quartz suspension into 16 male mice (weight 15–25 g.) 8 were given 2·5 mg. of cortisone acetate daily and 8 were kept as controls. This dosage was not well tolerated, as all those receiving it were dead within 17 days, the controls meanwhile showing no evidence of illness.

By the fifth day after the quartz injection, the powder in the peritoneal cavity had become clumped. In the control the clumps were firmly adherent to the peritoneal surface, while in the cortisone-treated mouse they were lying free within the sac. By the eleventh day there was an obvious macroscopic connective-tissue reaction around the quartz deposits in the control, but in the cortisone-treated animal nearly all the quartz was still lying free. These differences were still obvious at the seventeenth day. In the control at this stage, the quartz collections were surrounded by distinct fibrous capsules forming discrete nodules. Histologically the nodules were composed of cells, mainly fibroblasts and connective-tissue fibres. On the other hand, in the cortisone-treated mouse at the seventeenth day most of the quartz clumps were still free, while a few small collections which had become adherent to the peritoneal surface were practically acellular and not accompanied by any significant degree of fibrosis or other vital reaction (Fig. 1 and 1*a*).

*Rabbits.*—The 12 animals used weighed between 2400 and 2900 g. and were each given 2 ml. of the 10 per cent quartz suspension intraperitoneally. Six were given 20 mg. of cortisone daily by subcutaneous injection, while the other six which served as controls received instead a corresponding volume of the aqueous suspending agent. One cortisone-treated and one control animal, closely matched according to weight, were killed 15, 30, 45, 75 and 90 days after giving the quartz. One rabbit died on the seventeenth day.

By the fifteenth day the quartz powder had formed macroscopic aggregations. In the cortisone-treated rabbits most of the aggregations were adherent to the peritoneal surface, but some were free. In the control all aggregations were firmly adherent, and were associated with much more fibrosis than in the cortisone-treated animal. Histologically the difference was qualitative as well as quantitative. In the cortisone-treated rabbit, although there were a moderate number of fibroblasts, they were mainly plump and polygonal and were associated with only little reticulin or collagen formation. In the control, on the other hand, the fibroblasts were elongated and accompanied by the formation of many connective-tissue fibres (Fig. 2 and 2*a*).

After 30 days a peculiar and unexpected change was found within the peritoneal sac of the cortisone-treated rabbit. When the abdomen was opened it appeared to be filled with many pink gelatinous polypi in which were entangled

aggregations of quartz. Histologically this polypoid growth was found to consist, to a very large extent, of thin-walled blood-vessels. There were some mononuclear cells between the vessels but only a small amount of fibrous connective tissue (Fig. 3). The vessels lay in a copious structureless ground substance. The nature of the latter was not determined as it did not stain with toluidine blue, Best's mucicarmine stain or by the periodic acid-Schiff technique.

By the ninetieth day this redundant vascular overgrowth and its ground substance had become replaced by fibrous connective tissue, which was widespread and disorderly and bore no apparent relationship to the few quartz nodules sparsely scattered throughout.

The control animals showed none of these diffuse changes, but only nodular deposits of quartz surrounded by pearly white capsules of dense white fibrous connective tissue.

Histologically, also, even after 90 days there was a distinct difference in the reaction immediately around the quartz. In the cortisone-treated rabbit there was a thin fibrous capsule, the bulk of the quartz remaining acellular. In contrast, in the control animal the nodules were very cellular, and consisted to a large extent of the whorled collagenous fibrous tissue typical of silicotic nodules (Fig. 4 and 4*a*).

The cortisone dosage of 20 mg. daily over a long period did not appear to affect the general health of the rabbits and only one died. The animal that was allowed to survive for 90 days remained apparently healthy for that time and lost only 100 g. in weight, the control rabbit during the same time losing 50 g. There was no obvious macroscopic difference in the rate of healing of the laparotomy wounds between the cortisone-treated rabbits and the controls.

*Rats.*—Adult male rats of a mixed breed and weighing 160–190 g. were used. Each of 12 animals was given 0·5 ml. 10 per cent quartz suspension through peritoneal incision. Subsequently six were given 5 mg. cortisone acetate daily and six controls received an equivalent volume of the aqueous suspending fluid.

This dosage of cortisone seemed to be near the limit of toleration because several of the rats died during the experiment, the longest survival being 58 days.

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EXPLANATION OF PLATES.

FIG. 1.—Cortisone-treated mouse at 13 days. 1*a*: Corresponding control. The quartz nodule from the treated mouse is almost acellular, whilst that from the control is very cellular and overrun by fibroblasts. Haemalum and eosin. × 55.

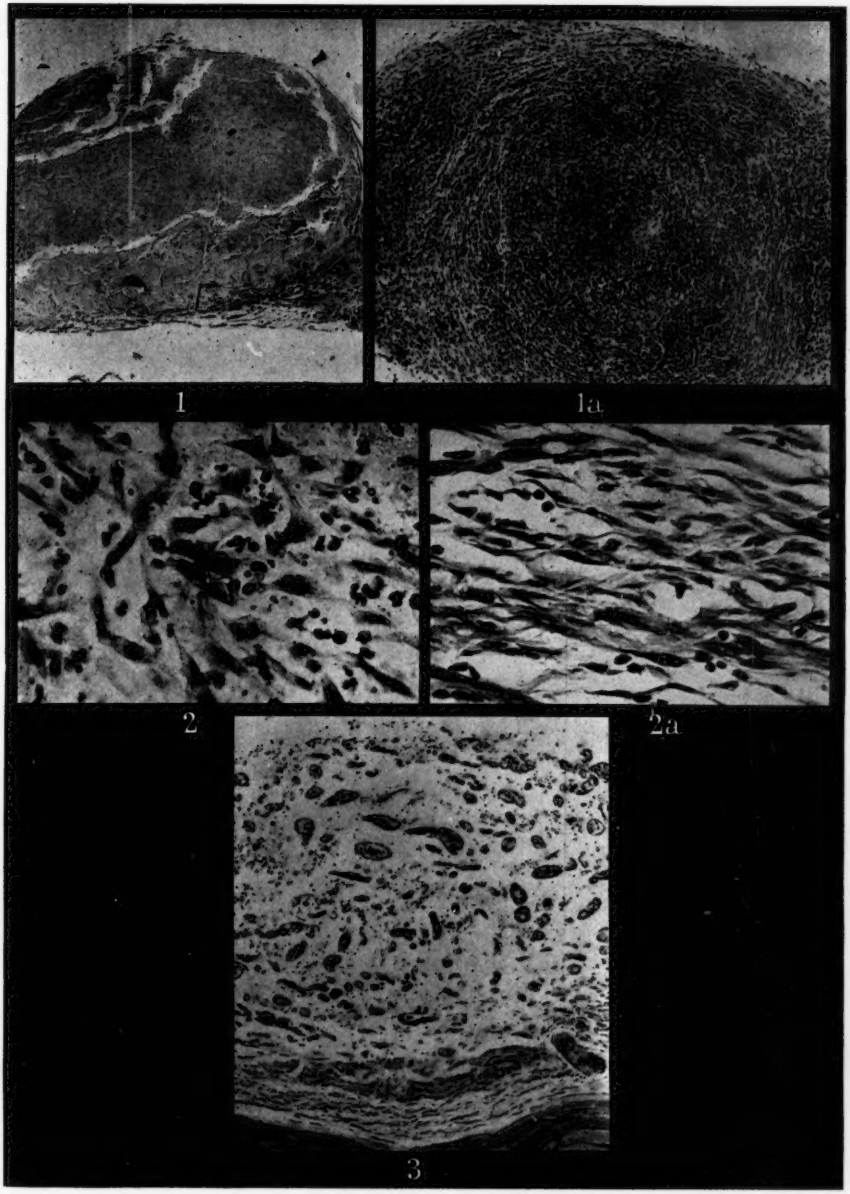
FIG. 2.—From the 15-day cortisone-treated rabbit. 2*a*: Corresponding control. In the treated rabbit the fibroblasts are plump and not associated with much new fibril formation. In the control the fibroblasts are elongated and accompanied by the formation of connective-tissue fibres. Haemalum and eosin. × 360.

FIG. 3.—Abdominal wall of 30-day cortisone-treated rabbit. The polypoid growths consist of many thin-walled blood-vessels in a structureless ground substance. Haemalum and eosin. × 40.

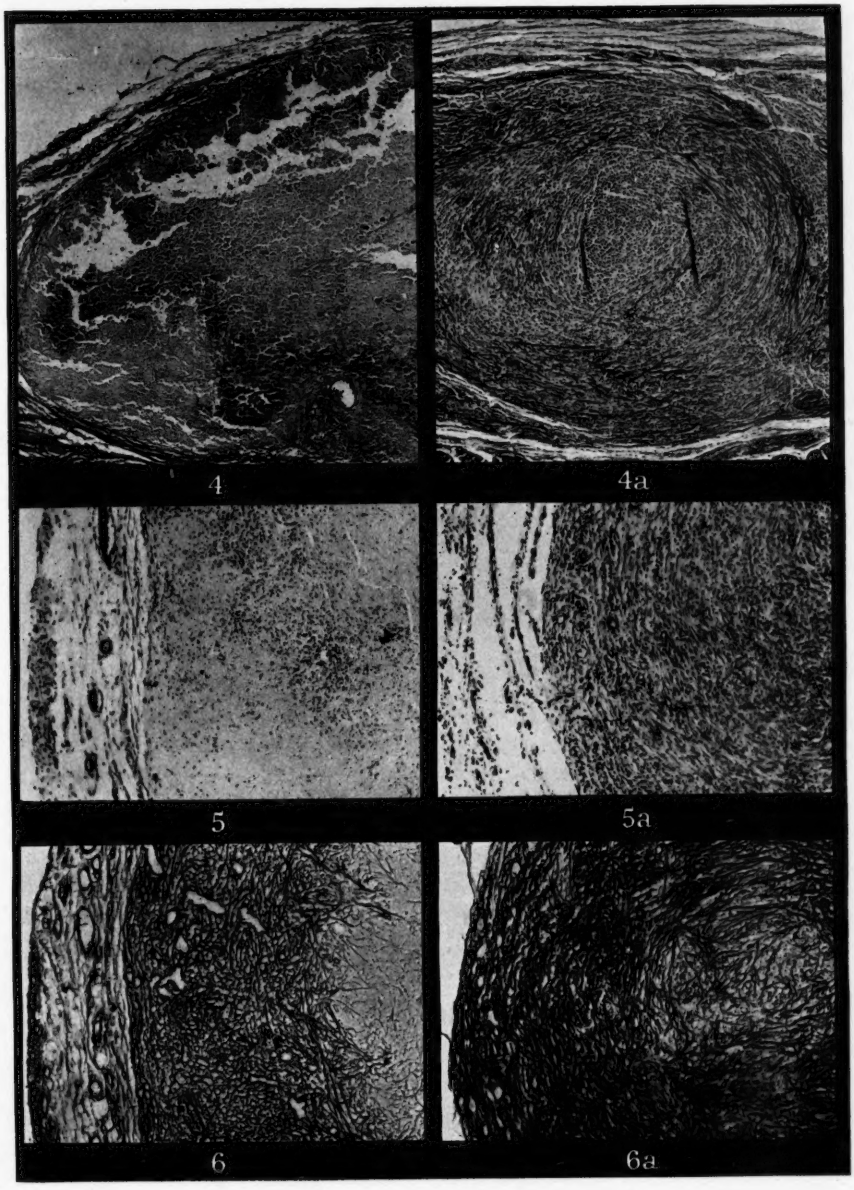
FIG. 4.—Quartz nodule from 90-day cortisone-treated rabbit. 4*a*: Corresponding control. The nodule from the treated rabbit is acellular and surrounded only by a very thin fibrous capsule. In the control the nodule is very cellular and replaced to a large extent by whorled collagenous fibrosis. Haemalum and Van Gieson. × 40.

FIG. 5.—Quartz nodule from a 25-day cortisone-treated rat. 5*a*: Corresponding control. In the treated rat only very few cells and blood-vessels have penetrated into the accumulation of quartz, whereas in the control the accumulation is overrun by cells, especially fibroblasts. Haemalum and eosin. × 60.

FIG. 6 and 6*a*.—Similar sections to Fig. 5 and 5*a*, but stained by silver impregnation. There are many connective-tissue fibres in both nodules, but they are thicker and more numerous in the control. × 60.




Magarey and Gough.




Magarey and Gough.

The control animals meanwhile remained healthy. The cause of death of at least two of the rats was extensive pulmonary aspergillosis.

When the lesions in the cortisone-treated rats were compared with those of the controls, it could be seen that the former showed some diminution and retardation in the formation of fibrous tissue, but the effect was less marked than in the mouse or the rabbit. The difference could nevertheless be well seen after 25 days. When stained with haematoxylin the quartz nodules of the cortisone-treated rats were far less cellular than in the controls (Fig. 5 and 5*a*). With connective-tissue stains, on the other hand, the differences present were less striking ; silver staining, for instance, revealed that fibres had grown into the quartz nodule in the cortisone-treated rat. In the control, however, the new fibres were thicker and more numerous and had overrun almost the whole of the nodule (Fig. 6 and 6*a*).

*Guinea-pigs.*—Each of 12 male guinea-pigs (weight 480–730 g.) received 1·0 ml. 10 per cent quartz suspension by intraperitoneal injection. Half of this number were given 10 mg. cortisone acetate daily, the other half being controls. Pairs of animals were killed at intervals up to a period of 30 days, the first pair only 5 days after the quartz injection.

At no stage was there any significant difference between the treated and control animals, for in both the quartz powder rapidly formed clumps which adhered to the peritoneal surface and became covered by a firm white fibrous capsule.

## DISCUSSION.

The large doses of cortisone used in these experiments suppressed the fibrosis induced by finely powdered quartz in the peritoneal cavity of the mouse, retarded its development in the rabbit and rat and had no effect in the guinea-pig. The relative resistance of the latter species to cortisone has been previously reported. Upton and Coon (1951), who studied the action of cortisone on wound healing, suggested that unlike that in certain other species, connective-tissue formation in the guinea-pig is reduced but little, if at all, by ACTH and cortisone.

Coste, Basset and Delaunay (1951) and Michael and Whorton (1951) have shown that cortisone reduces cellular infiltration in acute inflammation, and several groups of workers have shown that it retards the formation of granulation tissue during the healing of wounds. Nearly all published work has been based on short-term experiments only. Spain, Molomut and Haber (1950) for instance followed the effects of cortisone on wound healing in mice for 5 days. They found very little new capillary formation and only sparse fibroblastic proliferation during that time in treated animals as compared with controls. Ragan, Howes, Plotz, Meyer and Blunt (1949), using rabbits, showed that after wounding, cortisone strikingly depressed new growth of all the connective-tissue elements up to 8 days. Few if any new blood-vessels were to be seen and the fibroblasts lay in a compact arrangement in nests about old blood-vessels, whereas in the controls, fibroblasts were present throughout the wound and fibres had appeared between them. Bangham (1951), also investigating the effect of cortisone on wound healing, carried his observations on for 16 days. He found that the dosage of cortisone he used had no effect in guinea-pigs, that it retarded wound-healing in rabbits and had only a doubtful effect in rats.

Blunt, Plotz, Lattes, Howes, Meyer and Ragan (1950), using experimental fractures in rabbits, found little difference between cortisone-treated animals and controls for the first 3 or 4 days, but beginning with the fourth to fifth days the

differences were striking in so far as there was retardation of all phases of healing, but the period of observation in these experiments was only 8 days. The fibroblasts were noted to have rounded forms with bizarre arrangement of nuclear chromatin, and these features may well correspond with the plump and stellate forms we have found in the rabbit at 15 days. Blunt and his colleagues suggested that the failure of connective-tissue regeneration following fracture in cortisone-treated rabbits may be caused by an inadequate blood supply resulting from the failure of development of new blood vessels.

In our experiments the administration of cortisone was continued much longer, especially in the rabbit, in which it was given for 90 days. Throughout this period there was a great reduction in the amount of fibrous tissue formed in the quartz nodules of the cortisone-treated animals (Fig. 4 and 4*a*). As the object of the experiments was to discover the effects of cortisone on fibrosis over a long period, observations were not made for the first few days, but our findings in no way disagree with those of the above workers who used the shorter term experiments.

In the rabbit an unexpected feature made its appearance by about the thirtieth day. For the first 15 days after the administration of quartz powder there was not a great deal of reaction to its presence in the cortisone-treated rabbit, but in the control animal there was considerable fibrosis immediately surrounding the quartz deposits. By 30 days the peritoneal cavity of the cortisone-treated rabbit was almost filled by pink gelatinous material, which consisted of many thin-walled blood-vessels separated by a copious ground substance. At this stage there was no significant fibrosis in this oedematous-looking tissue. Only later did fibrosis appear, but it was present by 75 days and was diffuse and extensive by 90 days. It appeared to have little relationship to the few deposits of quartz sparsely scattered throughout.

These changes suggest various possibilities. It may be, as suggested by Blunt *et al.*, that cortisone first inhibits vascular proliferation. But in our experiments in the rabbit it would appear that after about 30 days of continuous treatment this inhibition is lost, and the blood-vessels then not only proliferate, but do so in a most disorderly and profuse fashion. This vascular proliferation is soon followed by fibrosis, which being possibly related to and controlled by the growth of the blood-vessels, is itself disorderly and profuse, thus producing the diffuse ragged fibrosis which had developed after 90 days of cortisone treatment.

Another possible explanation of the bizarre changes in the rabbit is that cortisone has an effect on the fibroblasts themselves, as indicated by the work of Barber and Delaunay (1951). The latter showed that the growth of fibroblasts in tissue culture was greatly inhibited by the presence of plasma from animals treated with cortisone. An even greater inhibition was obtained when cortisone itself was added to normal plasma. In our rabbits it may be that cortisone, having delayed the proliferation of fibroblasts and the maturation of collagen, thereby caused an abnormal accumulation of ground substance, and this subsequently became invaded by new blood-vessels and later fibroblasts. We cannot, however, be sure that this is the correct explanation. It may be that the failure to encapsulate the quartz by fibrous tissue allows diffuse irritation as the particles go into solution in the peritoneal fluid.

The initial retardation by cortisone of the development of granulation tissue

is clear from these experiments, but further investigation is needed to determine whether the later redundant fibrosis in the rabbit is also a cortisone effect.

SUMMARY.

Finely powdered quartz was introduced into the peritoneal cavity of mice, rats, rabbits and guinea-pigs and the effect of cortisone on the ensuing fibrosis was studied.

Cortisone inhibited fibrosis in the mouse, depressed it in the rat and had no effect in the guinea-pig.

The findings in the rabbit were anomalous. Fibrosis in the quartz nodules was greatly diminished by cortisone, but following a period of about 30 days there was great vascular proliferation in the peritoneal cavity followed by a disorderly and profuse fibrosis.

We are very grateful to Dr. G. Nagelschmidt for the quartz powder and to Mr. J. P. Napper for technical assistance.

The cortisone used in this work was provided from a generous gift made to the Medical Research Council by Merck & Co., Inc.

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# THE EFFECT OF CORTISONE ON THE REACTION OF THE MOUSE PERITONEUM TO QUARTZ

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CORTISONE exerts an effect on the formation of granulation tissue (Ragan, Howes, Plotz, Meyer and Blunt, 1949; Spain, Molomut and Haber, 1950); amongst its effects there is marked diminution of cellular elements, reduced formation of new capillaries, sparse proliferation of fibroblasts and relative lack of ground substance. Epithelialisation is unaffected. The effect is similar in some respects to that seen in vitamin C depletion (Penney and Balfour, 1949), especially in the absence of metachromatic ground substance. Cortisone delays the early inflammatory response in the skin of rabbits (Michael and Whorton, 1951), and greatly retards the macrophage response to intraperitoneal carbon (India ink) (Spain *et al.*), possibly by antihyaluronidase activity (Seifter, Baeder and Begany, 1949). The fibrogenic properties of quartz provide an obvious opportunity to examine those aspects of cortisone activity and the experiments detailed below describe the results obtained in mice.

## MATERIALS AND METHODS.

The material was D.R.C. quartz with a mean particle diameter of approximately 1μ. This was suspended in sterile normal saline in a concentration of 4 g./100 ml. The suspension was made immediately before use and sterilised for one hour at 100°C. Each mouse received 0·5 ml. (20 mg. quartz) intraperitoneally.

The cortisone used was the acetate (Merck) diluted with sterile saline, so that the injection (0·2 ml.) contained 0·4 mg. This was the standard dose for 14 days, reduced thereafter in Series B to 0·1 mg. daily.

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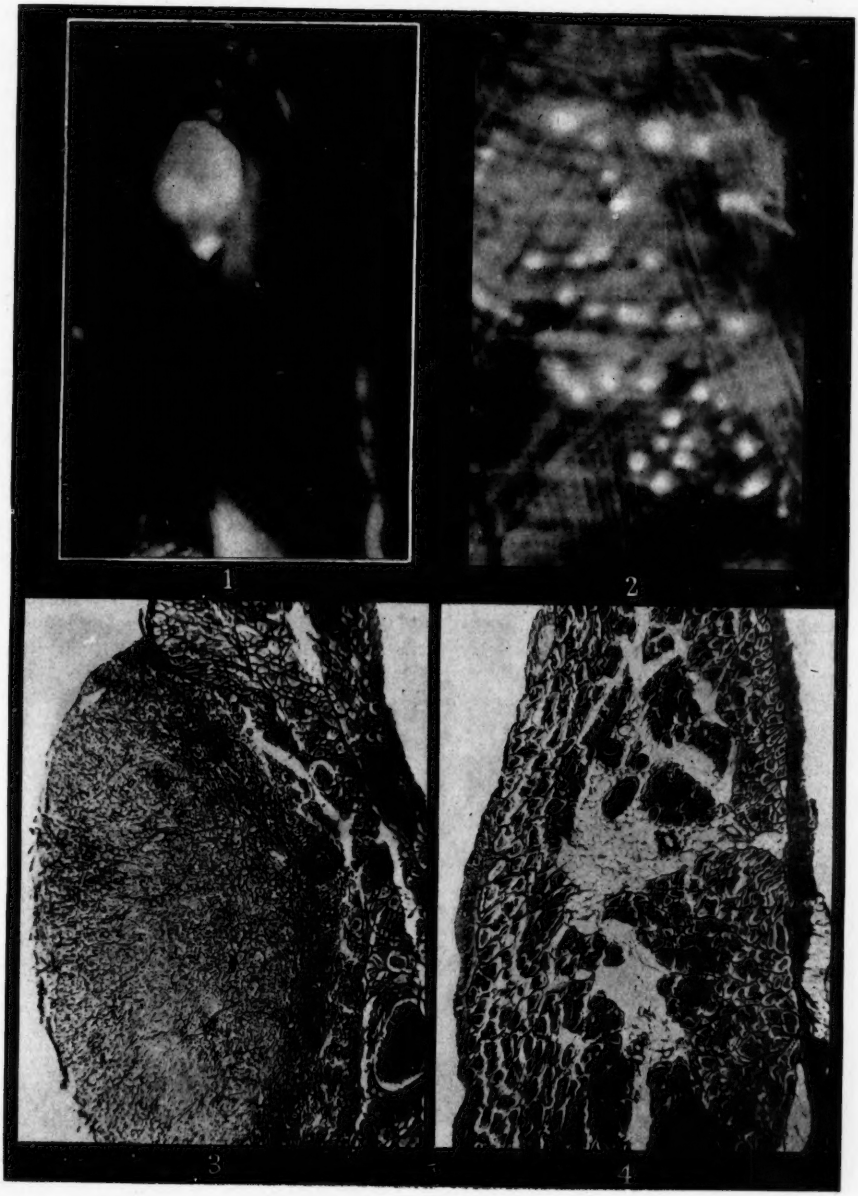
## EXPLANATION OF PLATES.

FIG. 1.—Heaped-up dust nodule on the peritoneal surface of the abdominal wall of a control mouse 91 days after administration of quartz. This type of lesion is not found in the cortisone-treated animals. × 5.

FIG. 2.—Thin fibrous membrane on the peritoneal surface of the abdominal wall of a cortisone-treated mouse killed 84 days after quartz injection. The membrane is visible because of slight nodularity. × 5.

FIG. 3.—Section of a proliferative dust nodule on the abdominal wall of a control animal killed 62 days after quartz injection. The muscle is atrophic. Reticulin stain. × 40.

FIG. 4.—Very thin collagenous membrane on the peritoneal (left) surface of the abdominal wall of a cortisone-treated mouse, killed 84 days after quartz injection. Reticulin stain. × 40.



Curran.

*Series A.*

Twenty mice received 0·4 mg. cortisone subcutaneously for 14 days. Four hours after the second injection 0·5 ml. (20 mg.) quartz suspension was injected intraperitoneally. The animals were killed 1, 2, 3, 5, 8, 10, 14, 21, 31, 48, 62, 84, 112 and 122 days after the quartz injection.

*Series B.*

Fifteen mice were given quartz, and cortisone as Series A for 14 days. Thereafter each received 0·1 mg. cortisone daily for 76 days. Animals were killed 1, 14, 21, 28, 31, 48, 62, 84, 112 and 122 days after the quartz injection.

*Series C.*

Fifteen mice received 20 mg. quartz intraperitoneally. After 90 days a 14-day course of cortisone (0·4 mg. daily) was given. Animals were killed 7, 31, 62, 84, 91, 92, 93, 94, 96, 98, 102, 106, 113, 129, and 158 days after the quartz injection.

*Series D* (Control).

Twenty-four mice were treated in all respects as Series A except that sterile saline was substituted for cortisone. The animals were killed 1, 2, 3, 5, 7, 9, 10, 14, 21, 31, 48, 62, 84, 91, 112, 122, 129 and 158 days after the quartz injection.

Omentum, mesentery, pelvic fat and portions of abdominal wall were removed at autopsy and immersed in Bouin's fixative for 18 hours. Paraffin sections (5$\mu$ in thickness) were stained by a variety of methods including haematoxylin and eosin, Heidenhain's iron-haematoxylin and van Gieson, Gordon and Sweets' method for reticulin, toluidine blue, thionin, and the periodic acid-Schiff method.

## RESULTS.

*Control Series D.*

The early reaction to quartz is seen in mice killed within a week of the injection. During this period there is usually a viscous peritoneal exudate, mainly polymorphonuclear in its cellular character for the first three or four days but predominantly mononuclear thereafter. At 18 hours most of the dust has aggregated into clumps which vary in size up to 4 mm. visible to the naked eye and notably adherent to the omentum, parietal peritoneum, pelvic fat and mesentery. In addition to those clumps the omentum is usually finely powdered with dust particles. Reticulin is formed in the nodules at an early stage. By the third day the smaller nodules show a reticulin network throughout their substance. In the larger clumps reticulin is limited to the periphery for some time. Organisation and fibrosis of the dust masses take place from the periphery. Coincident with the formation of reticulin, all the dust aggregations show a progressive encroachment by cells which resemble macrophages, but are functionally fibroblasts and which ultimately replace the entire nodule. This process of cellular invasion occupies several months, and is associated with the deposition of collagen in the nodules. Collagen is detectable in most cases by the 10th

day, but becomes progressively more dense until after several months typical silicotic nodules are formed. Metachromatic staining of the dust collections is noticeable in the earliest lesions and persists until fibrous replacement occurs.

*Cortisone Series A and B.*

These differed from the control series in several ways. The turbid exudate in the peritoneal cavity was usually less marked, though no difference was noted in the initial acute cellular response. Much free dust persisted in the peritoneal cavity of the cortisone mice up to 21 days, and there was an absence of prominent nodules on the parietal peritoneum. Instead of the heaped-up lesions of the abdominal wall, so characteristic of the controls (Fig. 1 and 3), the mice of Series A and B showed a very thin pearly fibrous membrane covering the abdominal wall. This diffuse fibrosis of the parietes was noted as early as the 8th day, but became more evident after several months, when occasionally it presented a fine nodularity (Fig. 2 and 4). The omental, mesenteric and visceral dust aggregations, however, appeared and behaved like those of the controls: reticulin and collagen were laid down in them at the same rate as in the control mice. Metachromasia was detected in the nodules.

*Cortisone Series C.*

Mice killed 90 days after receiving the quartz were seen to have silicotic nodules similar to those of the other series. No significant effect was noted in any of the animals up to two months after the cortisone injections.

## DISCUSSION.

The peritoneal cavity of the mouse was chosen for this study partly on account of our previous experience in testing a large series of dusts in mice, and partly because the mouse peritoneum yields true silicotic nodules, comparable in all respects with the human lesion, with fibrogenic dusts in three or four months. Probably on account of the *tâches laiteuses* those nodules are particularly evident in the omentum and mesentery, but the mouse has the additional advantage that the response of the parietal peritoneum is usually well-marked with the formation of large discrete nodules, and is similar to that described in the guinea-pig by Miller, Sayers and Yant (1934).

The dosage of cortisone was calculated as sufficient to produce the maximum effect consistent with the survival of the animals. D'Arcy Hart and Rees (1950) found that 0·5 mg. cortisone was tolerated by mice for 14 days, an amount which corresponds with Selye's (1949) dosage in rats. Magarey and Gough (1952) used 2·5 mg. daily, and this large dose killed all the mice in 17 days. In our experiments eosinophil counts showed that 0·4 mg. of cortisone produced an eosinopenia, and the adequacy of that daily dose was shown unequivocally by the marked loss of weight and condition of the animals, by the deaths of several, and by the presence of suprarenal atrophy in the survivors. For this reason in Series B the dose was reduced to 0·1 mg. daily after 14 days.

As compared with the controls, the initial cellular response of the cortisone animals was similar, but fixation of dust in nodules was greatly delayed. Thus in some mice, free dust was found in the peritoneal cavity as long as 21 days after the injection. In general in the cortisone animals the dust collections in the tissues appeared smaller and less numerous.

The most significant effect of cortisone appears to be related to the dispersal of the dust, and this is shown most noticeably on the parietal peritoneum. The control animals had the characteristic heaped-up nodular masses on the abdominal wall; those nodules are present within a day or two of the injection of quartz and grow rapidly. No large nodular formations of this type were seen in any of the cortisone animals. By contrast, in place of the nodules there appeared after a week a thin pearly membrane to represent the fibrous reaction. This membrane was not limited to the parietal peritoneum, but spread on occasion to the spleen, kidneys and liver. The appearance of the fully-developed lesion in the cortisone animals was sufficiently characteristic to lead to their identification at post-mortem examination. None of the animals showed the vascular polypi described by Magarey and Gough about 4 weeks after the injection of quartz in cortisone-treated rabbits.

No explanation can be offered for the greater dispersal of the dust in the cortisone animals. Our experience with ACTH indicates that it has a similar effect. Clearly it cannot be a local action as the cortisone was not administered intraperitoneally. In an attempt to elucidate the mechanism of this dispersal, mice injected with quartz were given hyaluronidase intraperitoneally without any effect on the silicotic nodularity: indeed in those mice the lesions on the abdominal peritoneum were most prominent. Metachromasia, especially as demonstrated by thionin, was present in all the lesions, cortisone and control, apparently to the same degree. Thus the dispersal of the cortisone lesions would not appear to be related to lack of metachromatic mucopolysaccharide to bind the dust particles.

In contrast to the diffuse lesion on the parietal peritoneum of the cortisone-treated mice, the changes in the omental and mesenteric quartz collections were similar to those of the control animals. Despite the delay in phagocytosis and the greater dispersal of the dust in the cortisone series, fibrogenesis started at the same time and progressed at the same rate as in the control series. The omental and mesenteric foci of dust were converted into densely fibrous nodules in several months. Even the mice with a daily maintenance dose of cortisone progressed like the controls. Consequently, no effect from a course of cortisone on mature lesions was anticipated; and in fact no change occurred in the animals thus treated up to two months after administration of the hormone.

SUMMARY.

In mice the response of the peritoneal cavity to quartz dust is modified by cortisone in several ways. The dispersal of the dust is accentuated, phagocytosis is delayed, and the usual heaped-up lesions on the abdominal peritoneum are replaced by a thin fibrous membrane. With the dosage used the ultimate fibrotic response to quartz is unaltered, and cortisone appears to have no effect on mature silicotic foci.

I wish to express my thanks to Professor G. L. Montgomery for his interest and criticism throughout this work. I am indebted to the National Coal Board (Scottish Division) for a grant towards expenses, to Messrs. Colin Stewart, Ltd., for the supply of D.R.C. quartz, and to Mr. Donald Hay, F.I.M.L.T., for the photographs and photomicrographs. My thanks are due also to the Medical Research Council for the supply of cortisone and ACTH.

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# A CRITICAL STUDY OF THE METHODS FOR HISTOCHEMICAL LOCALIZATION OF BETA-GLUCURONIDASES.

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QUANTITATIVE biochemical studies have revealed that β-glucuronidases are widely distributed in human and animal tissues. With pH optima at 4·5 and 5·2, these enzymes catalyse hydrolysis of the glucosidic linkage of naturally occurring and synthetic β-glucuronides. Fishman (1940) believed that β-glucuronidase was responsible for the synthesis of glucuronides *in vivo*, but the later work of Kerr and Levvy (1949) showed that this synthesis was unaffected by saccharate, which was found to be a specific inhibitor, at low concentrations, of the hydrolytic β-glucuronidase active at pH 5·2.

A close relationship has been demonstrated between the degree of proliferative activity of a tissue and its content of β-glucuronidase (Levvy, Kerr and Campbell, 1948). At the end of gestation, mammary, uterine and placental tissues all show high levels (Fishman and Anlyan, 1947). High levels of β-glucuronidase have been recorded for a variety of human malignant neoplasms; tissue biopsies of cervical carcinoma, for instance, are uniformly rich in glucuronidase by comparison with those from benign lesions of the cervix (Fishman *et al.*, 1951). Obviously, therefore, it is desirable that accurate histochemical localisation of the sites of enzyme activity should be achieved, but although two histochemical methods were proposed by Friedenwald and Becker (1948), their only reported use in the study of normal and pathological tissues appears to be that of Campbell (1949). Two further histochemical methods were suggested by Seligman (1949), giving a total of four possible methods. When the work which is reported here was begun, it was proposed to ascertain which of the four methods could most conveniently be applied to a systematic study of human neoplasms, and to conduct and report such a study. In fact, it proved necessary to spend a considerable time in evaluating the four methods, and modifying one of them so as to make it a practical procedure for the investigation of pathological material. What is presented, therefore, is a critical study of the histochemistry of the β-glucuronidases.

Two basic methods exist for demonstrating the presence of glucuronidase activity in the tissues. The first depends on the splitting of a soluble glucuronide to give an insoluble product which is deposited *in situ*. This product may be coloured in the first instance (Section 3, below), or it may be colourless or inadequately coloured from the microscopic point of view. In the latter case it

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is made visible by conversion *in situ* to a highly coloured substance (Section 2, below). The second method depends on the splitting of a soluble glucuronide to give a soluble product, which combines instantaneously with a third substance, present in the incubating medium, to give a water-insoluble final product. This is coloured in both cases (Sections 1 and 4, below), although in the second case the colour is altered by subsequent processes to make it more easily visible.

No matter which method is employed complete insolubility in water of the final product is a *sine qua non*, since any solubility will allow diffusion and therefore inaccurate localisation of enzyme activity. This process is well known in enzyme histochemistry. The coupling azo dye methods of Sections 1 and 2, and the non-coupling azo dye method of Section 3, are applicable to the demonstration of other enzymes by suitable alteration of the non-phenolic end of the molecule. The substitution of glucuronic by phosphoric acid, for example, allows them to be used for phosphatase studies (Menten, Junge and Green, 1944), and by acetic acid, for studies of non-specific esterases (Nachlas and Seligman, 1949) and choline esterases (Denz, personal communication).

The following have been suggested or employed as suitable substrates for the histochemical demonstration of β-glucuronidases :

(1) 1- and 2-naphthyl glucuronides.
(2) 6-bromo 2-naphthyl glucuronide.
(3) 1-(*o*-hydroxyphenylazo) 2-naphthyl glucuronide.
(4) 8-hydroxyquinoline glucuronide.

Although methods employing three of these four substrates have now been found unsatisfactory for the demonstration of glucuronidases, they are fully considered here for two reasons : First, they have been mentioned in the literature as histochemical substrates for the glucuronidases, sometimes as part of a complete method but often without further details. We hope to show that workers in this field can confine themselves, at the present time, to one method only. Secondly, techniques employing substrates of similar types are now in use for the demonstration of other enzyme systems besides the glucuronidases. The consideration of such methods is therefore of wider interest than would appear to be the case if the present negative findings are regarded simply from the glucuronidase point of view.

The coupling azo dye methods used in histochemistry divide themselves conveniently into three groups. In the first, which may be called the simultaneous or syn-coupling method, the soluble phenolic product of hydrolysis is allowed to combine at the instant of its release with a diazonium compound. A coloured insoluble azo dye is precipitated at the site of enzyme activity. In the second, or post-coupling type of method the phenolic product of hydrolysis is itself insoluble and is deposited at the site of enzyme activity. It is subsequently converted to a coloured azo dye by treatment with a diazonium salt in alkaline solution. In the third, or non-coupling azo dye method the phenolic reaction product is itself a coloured insoluble azo dye. It is precipitated at the site of its release by the hydrolytic enzyme. So much for the theory of these methods.

### *1. Use of 1-Naphthyl and Similar Glucuronides as Substrates.*

No complete method has been published using these substrates for the histochemical demonstration of β-glucuronidase. Seligman (1949) found 1-naphthyl

glucuronide unsatisfactory on account of difficulties in coupling with diazonium salts at pH levels in the acid range. We therefore tried to evolve a method of simultaneous coupling using this substrate and different diazonium salts, some of which should have coupled satisfactorily at pH 5·2 with any α-naphthol released by enzyme action. In no case was any deposition of an azo dye obtained in the tissues with incubation periods of up to 6 hours at 22°, since the substrate was insufficiently hydrolysed. Using the diazotate of 4-benzoylamino-2:5-dimethylaniline, however, and incubating at 37° for 16 hours, coarse deposits of a red azo dye were produced in a variety of tissues. Accurate localisation was impossible by this method. We also endeavoured to produce a satisfactory simultaneous coupling method with the glucuronides of azoresorcinol and 8-hydroxyquinoline, in both cases without much success. The limiting factor in these experiments was the slow rate of hydrolysis of the glucuronides by the amount of enzyme present in tissue sections.

### *II. Use of 6-Bromo 2-Naphthyl Glucuronide as Substrate.*

Because of his failure to achieve a successful simultaneous coupling method for glucuronidase, using naphthyl glucuronides, Seligman (1949) endeavoured to synthesise a suitable glucuronide for use in a post-coupling type of procedure. The requisite characteristics were possessed by 6-bromo 2-naphthyl glucuronide (Seligman and Rutenburg, 1950), and Seligman and Nachlas (1950) reported that a "satisfactory" histochemical method had been evolved. We therefore decided to test the properties of this substrate.

*Method of preparation.*

Moderately pure 6-bromo 2-naphthol was prepared, though containing traces of tin salts as impurities ('Organic Syntheses,' vol. 20, p. 18). It was administered by a stomach-tube as a dose of 2 g. in 80 ml. water to each of three adult male rabbits; the urine was collected from a metabolic cage during the ensuing 36 hours. The (alkaline) urine was acidified with a few drops of acetic acid and continuously extracted with ether for 16 hours. The ether extract was dried *in vacuo*, leaving a sticky brown residue which was dissolved in a minimal quantity of hot water and heated with animal charcoal; the solution was filtered while hot, and on cooling the filtrate yielded a pale brown precipitate. This was dissolved in a small volume of hot water, and to it was added 1 g. *p*-toluidine as a concentrated solution in warm water; on cooling a bulky white flocculent precipitate separated, which was collected by filtration and twice re-crystallised from hot water. It was finally dried on filter-paper at 37°, and appeared as glistening pinkish-white plates of *p*-toluidine 6-bromo 2-naphthyl glucuronidate. After thorough drying it melted over the range 175–178°, with simultaneous brown coloration. It gave no test for free naphthol when diazotised diorthoanisidine was added to a solution in veronal buffer at pH 9·2. After acid hydrolysis the naphthoresorcinol (Tollens) reaction was positive, as was the test for free naphthol. The yield was poor, amounting to less than 0·5 g., and no claim is made that the above represents the optimal method of isolation.

Seligman (1949) had suggested the use of brucine for the final purification, and he therefore used the brucine salt as his actual substrate. Following the method of Berenbom and Young (1951) we used *p*-toluidine in place of brucine,

and it was thus necessary to confirm that the *p*-toluidine salt was sufficiently rapidly hydrolysed by the enzyme. For this purpose the substrate solution was prepared by dissolving 30 mg. of crystalline *p*-toluidine 6-bromo 2-naphthyl glucuronidate in 100 ml. M/10 acetate buffer (pH 5·2) at 37°. An identical solution was used for the histochemical tests described below. A dilute watery extract of fresh rat liver was centrifuged and the nearly clear supernatant collected; a few drops were added to each of two tubes containing 5 ml. of substrate, and to one tube was added sufficient potassium hydrogen saccharate to make the concentration 0·0025 M (cf. Kerr, Campbell and Levvy, 1949). Each tube was incubated at 37° for 30 minutes; its contents were then made alkaline and a few mg. diazotised diorthoanisidine added. A bright blue colour developed at once in the first tube, while the saccharate-inhibited control remained colourless; it can therefore be assumed that substantial hydrolysis had been produced by a glucuronidase active at pH 5·2. It would of course be possible to prepare the glucuronide of the free base (6-bromo 2-naphthol) from the *p*-toluidine or brucine glucuronidate, but this manoeuvre is unnecessary, since the presence of *p*-toluidine or brucine introduces no complications.

*Histochemical application.*

Histochemical studies were made on adult male rat kidney, both fresh and after fixation in 10 per cent formalin at 4° for 2 hours; 20μ fresh or 10–15μ fixed sections were cut on the freezing microtome and incubated in substrate solution, both at room temperature and at 37°. To some preparations a few mg. of different stable diazonium salts were added at the commencement of incubation; the remainder, after incubation, were thoroughly washed in water, and exposed for one minute to freshly prepared cold aqueous solutions of a diazonium salt, stabilised at pH 8 by weak veronal buffer. A large number of stabilised diazonium salts was tested. These included the diazotates of 5-nitroanisidine, 4-chloro-*o*-anisidine, 4-benzoylamino-2 : 5-dimethoxyaniline and *o*-dianisidine for coupling at alkaline pH levels, and the diazotates of 2:5 dichloroaniline, *p*-nitroaniline, *o*-dianisidine, 4-chloro-2-nitroaniline and 1-amino-anthraquinone for

EXPLANATION OF PLATES.

FIG. 1.—Rat renal cortex. Disposition of 6-bromo 2-naphthol released enzymically (by alkaline phosphatase at pH 9·2). Coupled with Fast blue B salt. Note diffuse tubular staining, and staining of red cells, artery wall and elastica. × 145.

FIG. 2.—Rat renal cortex. True disposition of alkaline phosphatase as shown by Gomori's syn-coupling azo dye method. × 145.

FIG. 3.—Rat renal cortex. Disposition of β-glucuronidase by the original Friedenwald and Becker method. Poor localisation of Prussian blue deposits. Unfixed frozen section. Neutral red. × 120.

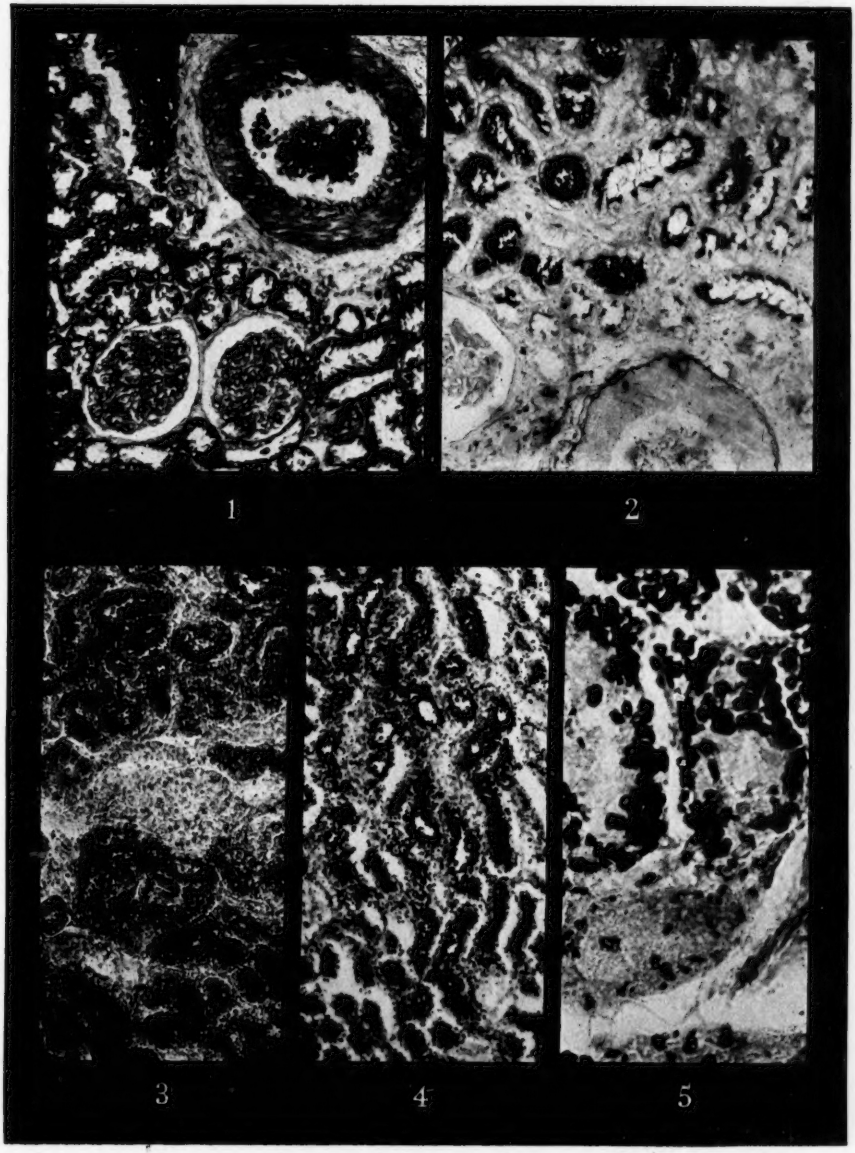
FIG. 4.—Rat renal cortex. Unfixed frozen section for comparison with Fig. 3. Shows the improvement produced by using ferric sulphate in place of chloride. Neutral red. × 115.

FIG. 5.—Identical section to Fig. 4. To show large size of Prussian blue deposits in the renal tubules which occur with unfixed material. × 400.

FIG. 6.—Rat renal cortex. Disposition of β-glucuronidase by the modified method using formalin-fixed frozen sections. Showing freedom from surface precipitation and small particle size. Upper left, distal convoluted tubules; lower left, proximal convoluted tubules. Neutral red. × 300.

FIG. 7.—Low-power view of complete section of rat kidney showing presence of β-glucuronidase in cortex and pyramid. Medulla almost free from enzyme. × 8.

FIG. 8.—Longitudinal section of renal pyramid. To show specific deposition of Prussian blue in the tubule walls. Modified method. Neutral red. × 235.

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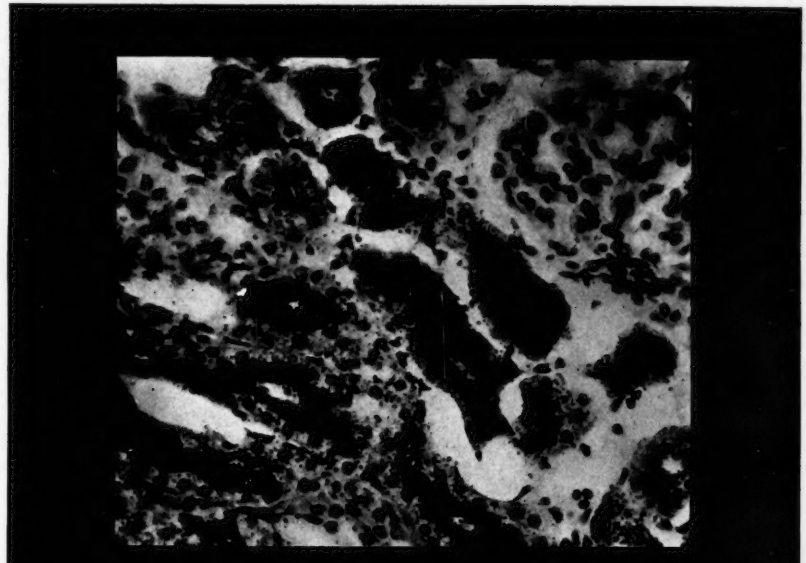

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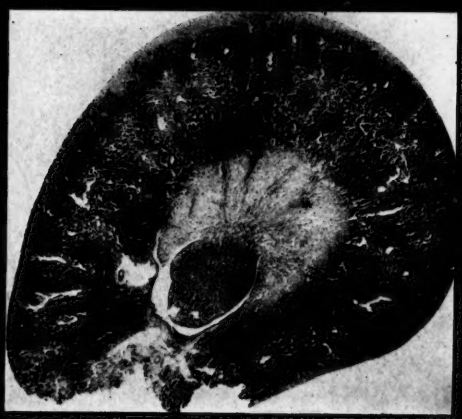

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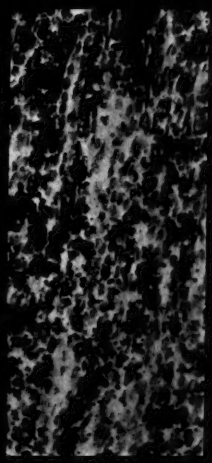

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coupling at acid levels. From the viewpoint of satisfactory colour production for histochemical purposes, the most suitable appeared to be *o*-dianisidine ("Brentamine Fast Blue B") and 4-chloro 2-nitroaniline ("Brentamine Fast Red 3GL"), both of which could be induced to couple with liberated 6-bromo 2-naphthol at pH 5·2.

*Results.*

Kidney sections incubated at 37° in the presence of a diazonium salt showed no evidence of dye deposition after 15 minutes; with longer incubations the dark brown staining of the tissue by decomposition products of the diazonium salt masked any other colour present. Tissues left at room temperature showed after an hour a trace of faint blue dye in the juxtamedullary convoluted tubules, though this was greatly obscured by brown staining. Tissues incubated in substrate solutions alone, and subsequently exposed to the diazonium salt, showed moderately intense staining after incubation periods of about one hour. The most highly coloured structures were the epithelial cells of the convoluted tubules (cytoplasm only), and also any attached fragments of fatty tissue; there was no staining of the glomeruli or collecting tubules. To the naked eye there was an intensely sharp delineation between the stained cortex and the colourless medulla. Several factors, however, made this result unacceptable as an indication of the precise location of enzyme activity, and these are set forth below:

(1) If after incubation the substrate solution was made alkaline and Fast blue B salt added, a bright blue colour instantaneously developed. It had been ascertained that the substrate was not prone to spontaneous hydrolysis under the conditions of the experiment, and diffusion of enzyme could not be demonstrated in formalin-fixed sections, so that this finding indicates the production of 6-bromo 2-naphthol by enzyme action and its subsequent diffusion in the incubating medium.

(2) Sections of kidney, incubated for periods of about 30 minutes in a dilute solution of 6-bromo 2-naphthol buffered at pH 5·2, showed, after coupling with a diazonium salt, an identical histological picture.

Fig. 1 and 2 illustrate the first point. Fig. 1 shows the diffuse staining of kidney structures by enzymically released 6-bromo 2-naphthol, subsequently coupled with Fast blue B salt. This picture was actually produced by the hydrolysis of 6-bromo 2-naphthol phosphate, by alkaline phosphatase at pH 9·2, in a cold acetone-fixed paraffin section of rat kidney. The strongest staining is in the cytoplasm of the tubular cells, but arterial walls and elastica especially, red cells, and to some extent the glomeruli, are also stained. Fig. 2 shows the picture produced in the same tissue by hydrolysis of sodium α-naphthyl phosphate simultaneously coupled, under otherwise identical conditions, with Fast blue B salt. Staining is confined to the brush borders of the tubules; the other structures are unstained. This is Gomori's (1951) modification of Menten, Junge and Green's (1944) technique for alkaline phosphatase. Phosphoric esters were chosen to illustrate this point, because the renal localisation of alkaline phosphatase is well established and because, in the case of the glucuronidases, no simultaneous coupling azo dye technique exists. The results shown in Fig. 1 and 2 really present a serious indictment of the post-coupling azo dye techniques as a whole. At all events it is clear that the liberated 6-bromo 2-naphthol or its *p*-toluidine salt is

sufficiently water-soluble for substantial diffusion to occur into the incubating medium. This occurs at pH levels on both sides of neutrality. In common with other naphthol derivatives the reaction product is then selectively taken up by certain tissue components to produce histologically acceptable pictures which, from the view-point of enzyme localisation, are merely artefacts. It is possible, using the salting-out principle, to depress the solubility of compounds such as 6-bromo 2-naphthol, so that in theory they should be precipitated in the tissues at their sites of production. Attempts to prevent diffusion by the use of high concentrations of salt (sodium chloride) in the substrate solution were not successful, however.

A method of the azo dye type, employing simultaneous coupling with a diazonium salt, would appear to give the most desirable approach to enzyme localisation, as it would largely remove the possibility of artefact due to diffusion of reaction products; the poor stability of most diazonium salts at low pH levels, however, necessitates very rapid enzymic hydrolysis, and the glucuronides so far tried do not meet this requirement. We conclude, from the above experiments, that 6-bromo 2-naphthol glucuronide is not a satisfactory substrate for the histochemical localisation of β-glucuronidase.

### *III. Use of* 1-(o-*Hydroxyphenylazo*)-2-*naphthyl Glucuronide as Substrate.*

A full method for the histochemical demonstration of β-glucuronidase was given by Friedenwald and Becker (1948), using the above compound as substrate. The method belongs to the non-coupling group where the reaction product is itself an insoluble azo dye, in this case bright red in colour. We prepared the compound exactly as described by these authors and used it in 50 mg./litre concentration in M/10 acetate buffer of pH 5·2, tissues being incubated at 37° for varying periods (1–24 hours). Kidney, liver, intestine and seminal vesicle of adult male rats were the tissues most often used, in the form of frozen sections from either fresh (unfixed) or formalin-fixed material. The latter was fixed for 4 or 24 hours at 4°. As controls, tissues were incubated after exposure to dry heat at 56° for 72 hours. From the findings of Sarkar and Sumner (1950) it can be assumed that such preparations were devoid of glucuronidase activity. Other control sections were prepared by including in the substrate solution potassium hydrogen saccharate at a concentration of 0·0025 M–0·005 M (Campbell, 1949). After incubation the sections were mounted in chrome glycerine jelly or in corn syrup, the dye being too soluble in organic solvents to allow dehydration and clearing. The keeping qualities of such sections were not good, the colour gradually diffusing into the mounting medium.

*Results.*

No substantial difference could be detected between test and control tissues; in each case the same general pattern of staining was produced, resembling that given by staining with 6-bromo 2-naphthol as described above. That is to say, there was selective staining of renal convoluted tubules and internal elastic lamina of arterioles and no staining of glomeruli or of collecting tubules. These findings are in agreement with those of Campbell (1949) on the failure of inhibition by saccharate. It appears that both the glucuronide (red colour) and its "insoluble" product of hydrolysis (also red) are capable of staining tissues; clearly

in the heat and saccharate-inhibited preparations the former is responsible for any staining observed. That the reaction product, 1-(*o*-hydroxyphenylazo)-2-naphthol, was also selectively adsorbed on to certain tissues was demonstrated as follows: fresh rat liver and kidney were ground with 0·1 M acetate buffer, pH 5·2, and the nearly clear supernatant, after centrifuging, was incubated at 37° for 30 minutes with a small amount of substrate solution. A red precipitate of free naphthol derivative settled out. This was extracted with benzene, dried *in vacuo*, and dissolved in 50 per cent ethanol. After immersion in this solution for several hours, formalin-fixed and heat-inactivated sections showed the typical dye pattern observed above. It was concluded that this method cannot be employed for the histochemical localisation of β-glucuronidase.

### *IV. Use of 8-Hydroxyquinoline Glucuronide as Substrate.*

A full method using this substrate was published by Friedenwald and Becker (1948). It is to be distinguished from the (unsuccessful) azo dye method, using the same substrate, which was attempted as part of the present study. It depends on the release of 8-hydroxyquinoline from its glucuronide, by the enzyme present in tissue sections, and the precipitation of this substance *in situ* as its ferric salt. Initial experiences with this method were disappointing, since it appeared highly capricious, and at best capable of giving only very coarse localisation of enzyme activity. Despite saturation of the substrate solution with ferric hydroxyquinoline, as recommended by the original authors, surface precipitation was found to be troublesome. The preparations appeared "dirty" as though the precipitate, which is finally visualised in the form of Prussian blue, had been deposited at random in some areas. Excessively large particle size made microscopic examination except at the lowest magnifications unprofitable, the deposits appearing as short needles or, more usually, as spherical crystals of up to 10μ in diameter (Fig. 5). The technically unsatisfactory nature of the preparations was increased by the necessity of using unfixed tissues, sections from which were seldom below 20μ thick. The illustrations given in their papers by Friedenwald and Becker (1948) and by Campbell (1949) were taken at very low magnifications, and it is difficult to see how Campbell was able to localise enzyme activity in the mitochondria of the renal tubular cells. The consistently negative results obtained with saccharate-inhibited controls, however, indicated that breakdown of the substrate was sufficiently specific for it to form the basis of a reliable method. In view of our failure to produce a satisfactory method with 6-bromo 2-naphthol, which also has this property, it was decided to concentrate on the Friedenwald and Becker technique. The procedure described below has several technical advantages; we claim that it is now sufficiently reliable for application to problems of human and animal pathology, and that a closer approach has been made to the accurate intracellular localisation of β-glucuronidase activity.

*The modified procedure.*

(1) Fix tissues in 10 per cent formalin at 4° for 4–48 hr. (optimum 4–12 hr.).
(2) Cut frozen sections into cold 0·1 M acetate buffer at pH 5·2.
(3) Incubate for 5–24 hr. at 37° in the following substrate solution:

Mix 13 ml. 0·01 M 8-hydroxyquinoline glucuronide in 0·1 M acetate buffer (pH 5·2), 13 ml. 0·01 M 8-hydroxyquinoline in 0·1 M acetate (pH 5·2),

9 ml. 0·03 M ferric sulphate and 1 ml. M acetate (pH 5·2). Incubate for at least 2 hr. at 37°. Centrifuge and store the supernatant at 4° in the dark. For use take 6 ml. of this solution, and for controls 6 ml. of the same solution containing 0·005 M potassium hydrogen saccharate.

(4) Wash briefly and mount the sections on albuminised slides, warming at 56° to ensure adherence (15 min.).

(5) Wash briefly and transfer sections to 0·5 M oxalate buffer (2·87 g. sodium oxalate, 0·47 g. oxalic acid in 100 ml. water) for 15 min.

(6) Wash and transfer to a fresh solution composed of equal volumes of 0·024 M potassium ferrocyanide, $K_4Fe(CN)_6\ 3H_2O$ (1 per cent) and N HCl (10 per cent) for 15 min.

(7) Wash and counterstain the nuclei with Mayer's carmalum (6–18 hr.) or with 1 per cent aqueous neutral red (1 min.).

(8) Wash, dehydrate in alcohol, clear in xylene and mount in a synthetic medium such as DPX (Kirkpatrick and Lendrum, 1941).

Tissues are fixed, as soon after removal as practicable, in cold 10 per cent formalin (at 4°) for any desired period up to 48 hours. That formalin fixation under prescribed conditions of time and temperature exerts relatively little inhibitory action on glucuronidase activity has been shown quantitatively by Seligman, Chauncey and Nachlas (1951). Employing the brucine salt of 6-bromo 2-naphthyl glucuronide as substrate, they found that 48 hours' fixation in 10 per cent formalin at 4° caused less than 20 per cent inactivation. In this regard glucuronidase was rather more resistant to inactivation than sulphatase, and still more so than non-specific esterase and acid and alkaline phosphatases. Within 30 minutes of cutting the sections are placed in the substrate solution. The latter differs from that of Friedenwald and Becker only in that 0·03 M ferric sulphate has been substituted for 0·03 M ferric chloride ; this is equally effective and much easier to handle. Ferric ammonium sulphate was also found to be a satisfactory alternative. It is of paramount importance that during its preparation the substrate solution should be incubated for at least 1½ hours at 37°. During this period the concentration of ferric hydroxyquinoline slowly reaches an equilibrium. After this stage centrifuging was found more convenient than filtration, which requires fine (No. 40) paper to obtain a sufficiently clear substrate solution for use. The substrate solution need not be used at once, since it keeps perfectly well at 4° ; it has been used successfully for up to 10 days after preparation. This stability saves both time and material. Furthermore, for the most convenient timing of operations, tissues can be left overnight immersed in substrate at 4° and incubated next morning. The incubation times which were found to be most satisfactory were of the order of 5 to 18 hours at 37°. A covered staining dish of capacity 6 ml., as used for fat stains on frozen sections, was found most convenient. The deposit of ferric hydroxide which invariably formed on the tissue during incubation was removed by exposure to oxalate buffer, as described by the original authors, and the Prussian blue reaction was finally developed according to their recommendations. Mounting the tissues on albuminised slides is conveniently carried out before the stage of treatment with oxalate buffer, but may be performed subsequently if so desired.

In view of the findings of Kerr, Levvy and Walker (1950) a trial was made of citrate buffer as a substitute for acetate in the substrate solution. There was

apparently no alteration of enzyme localisation or of intensity of deposition of ferric hydroxyquinoline. Deoxyribosenucleic acid (DNA) has been claimed by Bernfeld, Guarino and Fishman (1950) to activate purified preparations of ox spleen β-glucuronidase. The addition to 6 ml. of the substrate solution of 0·2 ml. of a preparation of DNA (containing 525 μg. of nucleic acid P per ml.) did not, in a single controlled experiment, increase the rate of deposition of ferric hydroxyquinoline.

*Results.*

For comparing the original and the modified methods, frozen sections of kidney obtained from male and female adult rats were used almost exclusively. The animals were killed quickly with chloroform or coal gas. In fresh frozen sections prepared by the older method, after 1–16 hours' incubation at 37°, a satisfactory amount of deposit was produced in the renal cortex. On microscopic examination this was observed to be very diffusely situated over the tubules and the crystal size was too large to permit reasonable localisation. Furthermore, diffuse deposits of Prussian blue were present in the medulla, and even in those parts of the cortex which appeared unstained to the naked eye (Fig. 3). When ferric sulphate was substituted for ferric chloride (Fig. 4) the general result appeared to be much clearer, and there was certainly less deposition of pigment away from the strongly stained regions. Crystal size was still very large, however, and accurate localisation impossible (Fig. 5). The small degree of enzyme inactivation produced by formalin is indicated by the fact that formalin-fixed tissues require the same incubation period as unfixed sections to achieve a comparable intensity of staining. The two most important differences between fixed and unfixed sections are first, that in the former surface precipitation in areas devoid of enzyme activity is much reduced, and secondly, that particle size is noticeably smaller in fixed material (Fig. 6). In both cases the control sections incubated in the presence of saccharate were entirely free from deposits. The general distribution of pigment in formol-fixed kidney is shown in Fig. 7. The clear midzone formed by the outer medulla, from which deposition is almost absent, is a regular feature. In the area of the collecting tubules in the pyramid the pigment is fairly sharply localised over the individual tubule walls (Fig. 8), and there is no reason to suppose that this is an artefact. Presumably the preponderance of thin loops of Henlë in the intermediate zone has something to do with this particular distribution of enzyme. Using 8-hydroxyquinoline glucuronide we notice especially high concentrations of enzyme in the proximal convoluted tubules (Fig. 6, lower left), where the pigment granules are concentrated over the luminal half of the lining cells, and over the brush border, overlapping into the lumen to join the pigment on the other side. In the distal convoluted tubules there is much less deposition of pigment (Fig. 6, upper left) and its distribution is mainly over the luminal aspect of the lining cells.

## Discussion.

Friedenwald and Becker adduced as evidence for the validity of localisation by their quinoline method the comparable picture obtained by using the azo dye method. The localisations which we found do not resemble those given by 1-(*o*-hydroxyphenylazo)-2-naphthyl glucuronide, and they are also entirely

different to those given by free naphthol and its derivatives after subsequent coupling. We consider that the modified quinoline method, with formalin-fixed tissues, gives an accuracy of localisation which is only limited by the large size of the pigment deposit. Formalin fixation appears to alter some local factor in the tissues, possibly surface charge, which operates in unfixed material to cause selective precipitation in certain areas. The probable effect of such local factors was recognised by Friedenwald and Becker, who compared surface precipitation to the formation of Liesegang rings. Little difference is seen between material fixed in formalin for 24 hours at 5° and that fixed for shorter times. There is, however, a tendency for the better fixed material to yield " cleaner " histological preparations, and this may be interpreted as due either to the prevention of enzyme diffusion by formalin fixation, or else as the manifestation of some inactivation at sites of low enzyme concentration. A sex difference in the renal glucuronidase activity of mice was recorded by Kerr, Campbell and Levvy (1949), and this was recognised histochemically by Campbell (1949). In this study it has been confirmed that, in the strain of rats employed, male kidney tissue shows considerably higher glucuronidase activity in the cortical tubules than does female material, when they are simultaneously incubated in the same substrate solution for the same time.

## Summary.

(1) Four methods, or more accurately, four substrates have been proposed as the basis of histochemical methods for demonstrating β-glucuronidases. These are the glucuronides of 1- or 2-naphthol, 6-bromo-2-naphthol, 1-(*o*-hydroxyphenylazo)-2-naphthol, and 8-hydroxyquinoline.

(2) A comparative study of all these substrates (except 2-naphthol glucuronide), and some additional ones, has been made.

(3) Attempts to achieve accurate localisation of glucuronidase, using all four types of substrate, and simultaneous, post-coupling or non-coupling, azo dye techniques, have shown that these methods are not yet suitable for histochemical application.

(4) Only the 8-hydroxyquinoline glucuronide method of Friedenwald and Becker has been found free from the theoretical and practical objections to the other methods. The original method, however, was not found satisfactory in application.

(5) A modified method, using formalin-fixed frozen sections at 10–15μ together with a modified incubating medium, gives consistent results in a variety of tissues. The full method, which is considered suitable for application to pathological material and problems, is given in the text.

We are grateful to Professor R. T. Williams for a supply of azoresorcinol and 8-hydroxyquinoline glucuronides, to Professor Leslie Young for a sample of 1-naphthyl glucuronide, to Professor E. J. King for 6-bromo-2-naphthyl phosphate, and to Imperial Chemical Industries Ltd. for a number of stabilised diazonium salts.

The photomicrographs were taken by Mr. E. V. Willmott, F.R.P.S.

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# STUDIES OF THE MINERAL REQUIREMENTS OF MAMMALIAN CELLS*

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FOR many years tissue culture has offered an attractive field to those interested in cell and tissue metabolism. That results have been somewhat meagre is due in part to the fact that, despite numerous attempts, no synthetic or completely defined medium has yet been devised which will support continuous cultivation of cells. For this purpose it is still necessary to use complex media. These usually contain a body fluid such as serum, and often extracts of tissue, this tissue usually being embryonic. This paper records an attempt to discover a technique whereby the requirement of the cell for one group of nutritional components could be studied using such a complex medium. This has been essayed by the partial removal of minerals from a protein-containing medium, first by chelation, and secondly by the use of ion exchange resins. Minerals removed have been subsequently replaced in part or whole by reagent grade chemicals or by relatively pure chemicals.

## MATERIALS AND METHODS.

### *Tissue culture technique.*

The roller tube method described by Gey and Gey (1936) has been used. Stock cultures of the cells employed have been maintained in Pyrex test-tubes lined with an embedding culture medium consisting of chicken plasma 4 parts, human placental cord serum 4 parts, beef embryo extract 1 part and balanced salt solution (Gey) 1 part. The supernatant liquid medium was of similar composition but without plasma. Balanced salt solution (B.S.S.) had the following composition: $Na_2HPO_4.12\ H_2O$, 0·603 g.; $NaHCO_3$, 0·5 g.; NaCl, 16·0 g.; $MgCl_26H_2O$, 0·42 g.; $KH_2PO_4$, 0·050 g.; KCl, 0·75 g.; $CaCl_2$, 0·55 g.; Dextrose solution 10 per cent, 40 ml.; Water to 2000 ml.

For all experimental work the stock cultures have been subcultured to tubes containing only the liquid medium with no chicken plasma, the cells being grown

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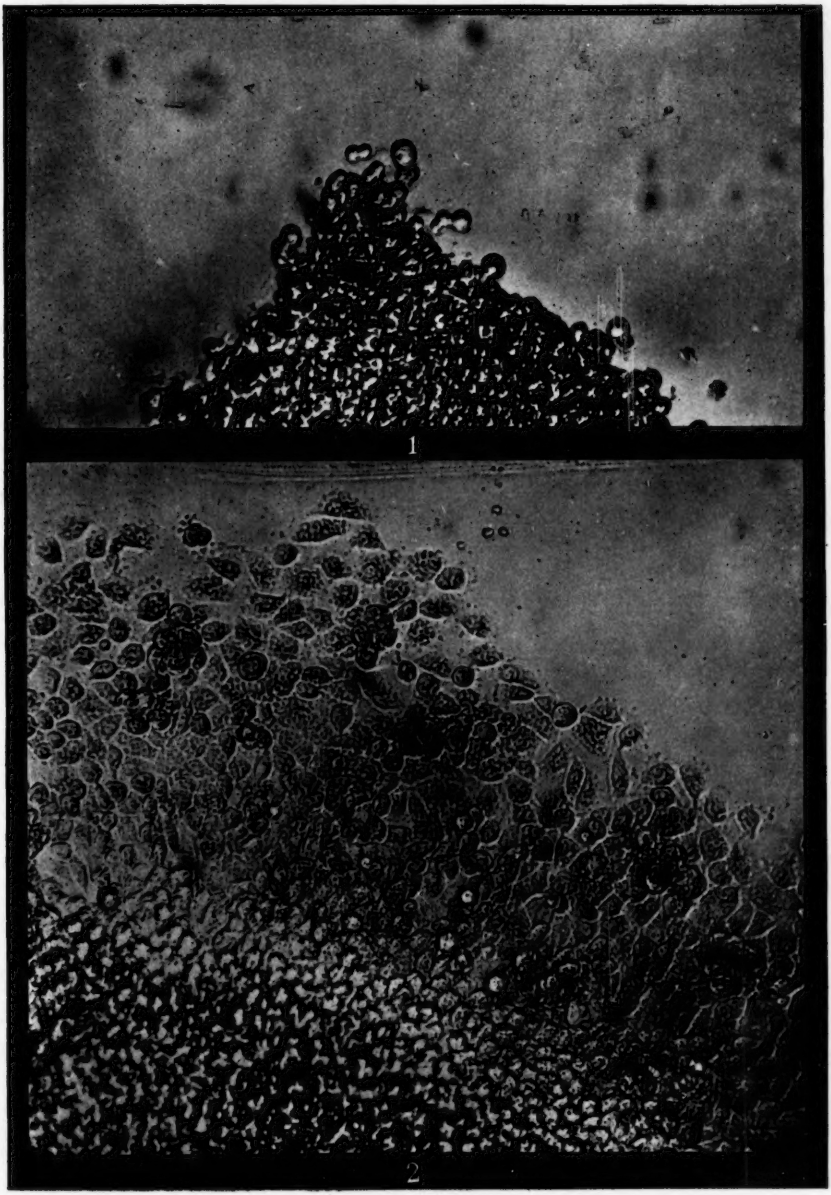
EXPLANATION OF PLATE.

FIG. 1.—A fragment of a colony of A.R.F. newly planted on a roller tube wall.

FIG. 2.—Appearance of the colony of A.R.F. after 24 hours' growth.



Shooter and Gey.

directly on the glass wall. Fragments of these cultures have been used for titrations, the cells again being grown directly on glass in the experimental fluid medium.

*Cells used and measurement of cell growth.*

A strain of cells derived 13 years ago from scrapings of the articular ridges of the femur of a rat (A.R.F.) or a strain of the Walker rat sarcoma No. 319 have been used. Cell growth over 24 hours was assessed by direct microscopic observation. Fig. 1 illustrates the appearance of a fragment of a colony of A.R.F. newly planted on a roller tube wall, Fig. 2 the appearance of the fragment after 24 hours' growth. In titrations it was assumed that where test colonies resembled control colonies in size, density and general appearance of the outgrowth, the nutritional requirements of that colony were temporarily satisfied.

*Reagents.*

Reagent grade chemicals were those of Merck & Co. The relatively pure chemicals used (except the calcium salt) were " Specpure " reagents purchased from Johnson Matthey.

Ion exchange resins were supplied by Rohm & Haas. Amberlites IRC 50 and XE 64 (a small particle version of IRC 50) were used for treatment of the medium ; Amberlites IR 120 and IRA 400 combined as a monobed were used for the preparation of metal-free water.

*Cleaning of glassware.*

All glassware required in a metal-free state was treated as follows : (1) Washed with soap and water ; (2) autoclaved containing a solution of a detergent and 0·1 per cent versine ; as soon as the pressure reached 15 lb. it was reduced to normal and the autoclave opened ; (3) rinsed with tap water ; (4) rinsed 10 times with metal-distilled water ; (5) rinsed 10 times with de-ionised water ; (6) autoclaved at 15 lb. for 30 minutes with de-ionised water.

## EXPERIMENTAL.

### 1. *Attempts to Rid Culture Media of Minerals Using a Chelating Agent.*

Citrate forms soluble chelate complexes with many elements, thus rendering them unavailable to some organisms (Hutner, 1948). The addition of sodium citrate in increasing amounts to the culture medium led to a progressive decrease in growth of the colonies until a point was reached at which no growth took place. This effect appeared to be mainly due to the removal of available calcium and magnesium, since, while addition of either of these two elements alone failed to restore growth, the addition of both together in appropriate amounts restored full growth for 24 hours. Under these conditions the addition of further citrate to the medium required the addition of more calcium and magnesium before normal growth was restored. An excess of magnesium was therefore provided, and the amount of calcium required for increasing amounts of citrate plotted to see if by extrapolation of these values to zero citrate an estimate of the basic requirements for calcium could be made. An example of this procedure is shown in Fig. 3. It was soon evident that only a most general approximation could

be made, and since calcium was present as ionised calcium, calcium bound to protein and as a citrate complex, it was probably not justifiable to attempt to extrapolate plotted values as a straight line.

### 2. *Attempts to Rid Culture Media of Minerals Using an Ion Exchange Resin.*

Amberlite IRC 50, a synthetic kation exchange resin, on account of its weak acid characteristics, can be easily buffered and, for this reason, has some advantages for use with serum. In early experiments medium consisting of human placental cord serum 4 parts and beef embryo extract 1 part was passed through

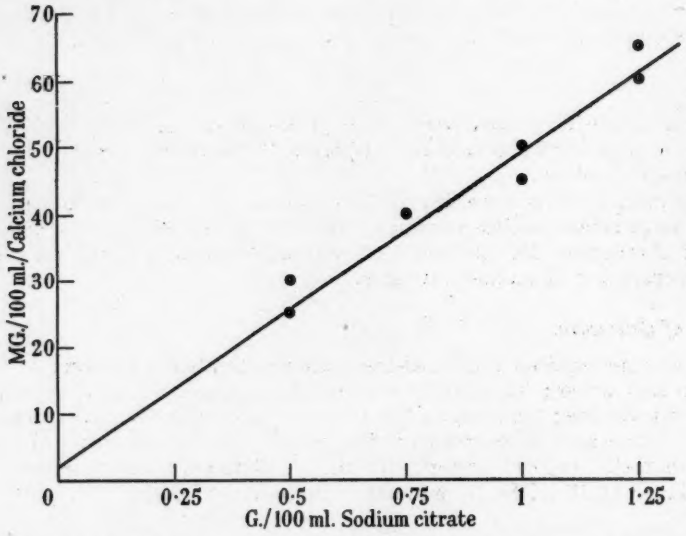



FIG. 3.—Amounts of calcium chloride required for full 24 hours' growth of A.R.F. with increasing concentrations of sodium citrate. Magnesium supplied in excess.

a short column of this resin buffered at pH 7·4 with a sodium and potassium phosphate buffer. The effluent for this column was found to contain sodium 136 milliequivalents per litre, potassium 5·8 milliequivalents per litre and calcium less than 0·77 mg. per cent.

Cultural results with this medium are shown in Table I.

TABLE I.—*Growth of A.R.F. with Treated Medium.*

| Medium. | Result. |
|---|---|
| Treated medium + water . . . . | No growth. |
| ,, ,, + B.S.S. . . . . | Full 24-hour growth. |
| ,, ,, + B.S.S.-Ca and Mg . . | No growth. |
| ,, ,, + Ca and Mg . . . | Full 24-hour growth. |

The addition of either calcium or magnesium separately to the medium failed to restore full growth, and it therefore became possible to titrate the requirement of the cell for calcium in the presence of varying amounts of magnesium, and the requirements for magnesium in the presence of varying amounts of calcium. This process is illustrated in Fig. 4 and 5, in which each point represents the end point of one or more titrations.

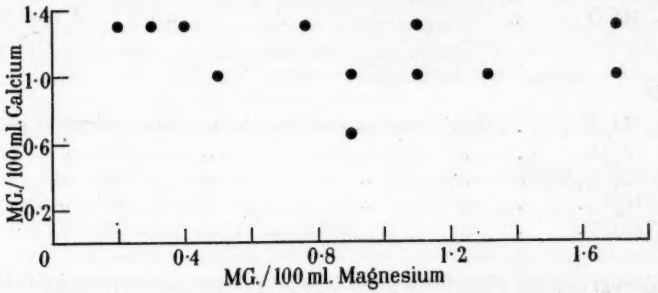



FIG. 4.—Minimum amounts of calcium needed for full 24 hours' growth of A.R.F. at varying concentrations of magnesium. Each point represents the end-point of one or more titrations.

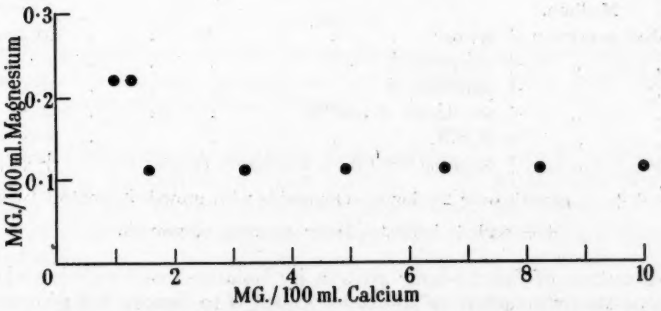



Fig. 5.—Minimum amounts of magnesium needed for full 24 hours' growth of A.R.F. at varying concentrations of calcium. Each point represents the end-point of one or more titrations.

This medium re-constituted with calcium and magnesium was adequate for 24 hours only. After this time little further growth took place, while the control colonies grew steadily in size. Since it was probable that other kations besides calcium and magnesium had been removed, it was decided to carry out a more vigorous extraction of the medium and to use materials free from contaminating traces of minerals.

Further batches of media were prepared by passage through IRC 50 buffered with a sodium phosphate buffer, followed by shaking with XE 64 in a beaker, the final product being sterilised by filtration through a sintered glass filter. From this stage the glassware used was chemically clean. The chemicals employed were relatively pure and were made up in de-ionised water.

Reconstitution of the treated medium was now attempted using solutions A and B which were designed to contain essential minerals.

*Solution A.*

| | | | | | |
|---|---|---|---|---|---|
| $Ca(NO_3)_2$ | . | final concentration of element in medium | | | 10 mg. per cent |
| $MgSO_4 7H_2O$ | . | ,, | ,, | ,, | 3 mg. ,, ,, |
| $KH_2PO_4$ | . | ,, | ,, | ,, | 15 mg. ,, ,, |
| $CuSO_4.5H_2O$ | . | ,, | ,, | ,, | 1 part per million |

*Solution B.*

| | | | | | |
|---|---|---|---|---|---|
| $MnSO_4 7H_2O$ | . | final concentration in the medium | | | 0·6 parts per million |
| $ZnSO_4 7H_2O$ | . | ,, | ,, | ,, | 0·6 ,, ,, ,, |
| $(NH_4)_6Mo_7O_{24}4H_2O$ | | ,, | ,, | ,, | 0·5 ,, ,, ,, |
| $FeSO_4 7H_2O$ | . | ,, | ,, | ,, | 2·0 ,, ,, ,, |
| $CoSO_4 7H_2O$ | . | ,, | ,, | ,, | 0·5 ,, ,, ,, |

The cultural results with these solutions and others are shown in Table II.

TABLE II.—*Growth of A.R.F. in Medium which had been Treated with IRC 50 and XE 64.*

| Medium. | Result. |
|---|---|
| Treated medium + water . . . . . | no growth |
| ,, ,, + solution A . . . . . | + |
| ,, ,, + solution B . . . . . | no growth |
| ,, ,, + solutions A and B . . . . | ++ |
| ,, ,, + B.S.S. . . . . | +++ |
| ,, ,, + normal serum + solutions A and B . | +++ |

+++ = growth over 24 hours comparable with growth in control tubes.

++ and + indicates lesser amounts of growth.

The restoration of full 24-hour growth by balanced salt solution alone made it improbable that the failure of solutions A and B to restore full growth was due to the removal from the medium of either lysine or arginine, or of growth factors, and this was confirmed by the lack of improvement when these substances were added in addition to solutions A and B.

The addition to the medium of solutions A and B minus either calcium, magnesium or potassium resulted in no growth.

## DISCUSSION.

The ability of ion exchange resins to remove minerals from tissue culture media without damaging the medium, except in so far as nutrients are extracted, appears to provide a way by which some of the mineral requirements of cultured cells can be determined. Resins cannot be expected to remove completely elements firmly bound to protein, and the degree of removal would seem to depend on the ability of the bound elements to ionise to replace already ionised components as they are removed. How far this is of importance in nutritional

studies is uncertain, since protein-bound minerals are not necessarily available to the cell.

More effective chelating agents such as 8-hydroxyquinoline would effect a more complete removal than the citrate used, but they have as yet not been employed, since it is not easy to extract the chelate complex from the medium without damaging other constituents.

In the results obtained so far, the failure of solutions A and B to restore full growth in the treated medium is perplexing, since these solutions contain the elements generally accepted as necessary for growth. It is tempting to think that some other element or elements normally present in balanced salt solution as contaminants of its reagent grade constituents are necessary for growth, but there is as yet no evidence for this.

All the results reported here have been obtained from 24-hour cultures, and the method of measurement is not applicable for any longer period of growth. Experiments are now in progress in which it is assumed that if a strain of cells can be carried in culture with repeated subcultures for 100 days, the nutritional requirements of that cell must be satisfied.

Only two cell types have been used so far, but there appears to be no reason why these methods should not be applied to other normal and malignant cells, and particularly to cell cultures carrying viruses and to strains of normal and malignant cells maintained in this laboratory which were derived from the same normal cell.

SUMMARY.

A method is described whereby the requirements of cultured cells for minerals may be studied. Calcium, magnesium and potassium have been shown to be essential for growth of one strain of cells.

The authors wish to express their indebtedness to Dr. Arnold R. Rich for his advice during the course of these studies. They would also like to thank Miss J. King and Miss O. Owens for their assistance. In the case of one author (R. A. S.) this work was done during the tenure of a Rockefeller Travelling Fellowship in Medicine.

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